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The Biosynthesis of Pseudomonic Acid

by

Philip James Donlevy

A thesis submitted to the University of Bristol, in accordance with the requirements for the degree of Doctor of Philosophy in the School of Chemistry, Faculty of Science.

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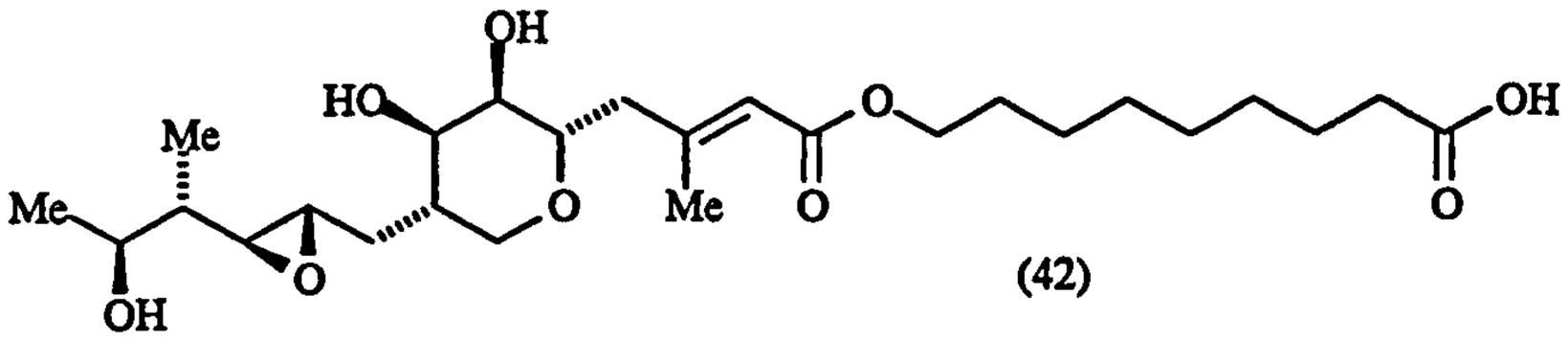
September, 1996.

ABSTRACT

Pseudomonic acid (42) is the major metabolite isolated from *Pseudomonas fluorescens* NCIB 10586, and is the active component in the topical skin cream Bactroban.



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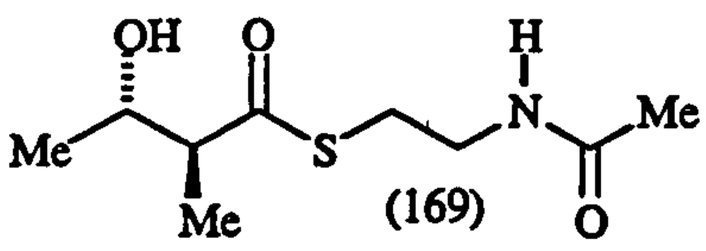
Previous biosynthetic studies have shown pseudomonic acid to be mainly polyketide in origin, possibly formed from a condensation of two distinct precursors: 9-hydroxynonanoic acid (69), the origins of which are unknown, and monic acid (50), which has been shown to be polyketide in origin. The aim of this research was to further investigate the biosynthetic pathway to pseudomonic acid.

Synthetic routes to a range of possible starter units of 9-hydroxynonanoic acid were developed, including the synthesis of [1,2-$^{13}C_2$]malonate, and [1,2-$^{13}C_2$]-3-hydroxypropionate, together with the corresponding singly labelled versions. Of particular importance was the synthesis of 9-hydroxynonanoic acid in $^{13}C_2$ labelled form. Our approach was based on the use of the Horner-Wadsworth-Emmons olefination of 7-hydroxyheptanal (147), and was flexible, thus allowing the synthesis of both [1,2-$^{13}C_2$]-9-hydroxynonanoic acid and [2,3-$^{13}C_2$]-9-hydroxynonanoic acid.

To elucidate information on the monic acid moiety, synthetic routes to the putative diketide (169) and triketide precursors (170) were developed.



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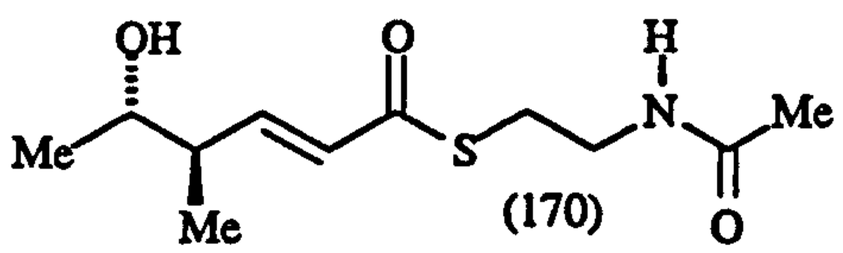

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Synthetic routes to the NAC thioesters of [1,2-$^{13}C_2$]monic acid A and monic acid C have also been investigated, in order to probe the later biosynthetic stages.

Initial feeding studies of acetate, propionate, butyrate, and malonate into *Pseudomonas fluorescens* strains PF3/R and NCIB 10586 have been ineffective in producing any information on the biosynthetic pathway. On feeding these precursors, pseudomonic acid production was blocked, and no significant incorporation of isotopic label was detected in pseudomonic acid.

To Mum, Dad, Neil and Lynn

ACKNOWLEDGEMENTS

I would like to thank Professor Tom Simpson for his help and advice during the past three years. I would like to thank Dr. Alan Moodie for similar support during my industrial placements. Financial support from SmithKline Beecham is gratefully acknowledged.

Thanks to Dr. Chris Willis for advice during the project, to Dr. Esi Bardshiri for work on the 9-hydroxynonanoic acid side-chain, and to Corinna Prengel for work on the monic acid intermediates.

Thanks also to the staff in the School of Chemistry for technical support, especially Jo Rhodes, and Rose Sylvester for nmr spectroscopy, and Dr. Ken McNeill for mass spectrometry.

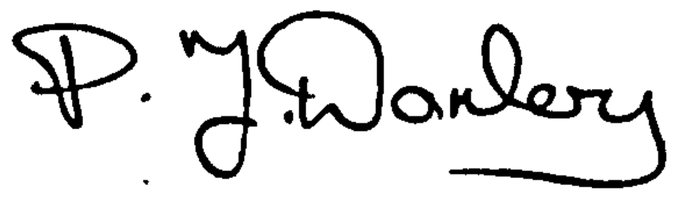
Many thanks to all past and present members of the Simpson and Willis groups, and to Mark, John, Mark, Louise, Matt, John, Sue, Nicola, Kate and Tim for making my time at Bristol most enjoyable. Most of all, thanks to Susie and Doug for keeping me sane!

I would like to thank my parents for supporting me throughout the whole of my education, and giving me the chance in the first place.

Finally, to Ruth. Without your help, I would never have got through the last four years. Thanks for being there all of the time, and listening day and night to all my gripes and groans about my PhD, Churchill, Medicine, and writing this thesis!

DECLARATION

The work described throughout this thesis was carried out solely by myself in the School of Chemistry, University of Bristol, and the Biotechnology Department, SmithKline Beecham Pharmaceuticals, Worthing under the supervision of Professor T. J. Simpson and Dr. A. Moodie, respectively, between October 1992 and September 1995. Except where indicated, by reference, the work is original and has not been submitted for any other degree.



Philip J. Donlevy

September 1996.

ABBREVIATIONS

The following abbreviations are used within the text:

acetyl CoA	acetyl coenzyme A
ACP	acyl carrier protein
α_D	optical rotation
br	broad
Bu	butyl
c	concentration
CI	chemical ionisation
CoA	coenzyme A
D	deuterium
d	doublet
dd	doublet of doublets
dt	doublet of triplets
δ	chemical shift
DCC	N,N'-dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DHP	dihydropyran
DIBAL-H	diisobutyl aluminium hydride
DMAP	4-dimethylaminopyridine
DMF	N,N'-dimethylformamide
DMSO	dimethylsulphoxide
EDCI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
EE	(1-ethoxy)ethoxy
EI	electron impact
Enz	enzyme bound intermediate
Et	ethyl
FAB	fast atom bombardment
FAS	fatty acid synthase
HMDS	1,1,1,3,3,3-Hexamethyldisilazide
HMPA	hexamethylphosphoramide
HSNAC	N-acetylcysteamine
HPLC	high pressure liquid chromatography
Hz	hertz
J	coupling constant
LDA	lithium diisopropylamine
lit.	literature
m	multiplet

M^+	molecular ion
mp.	melting point
m/z	mass:charge ratio
max	maximum
Me	methyl
nmr	nuclear magnetic resonance
υ	frequency
PDC	pyridinium dichromate
PEP	phosphoenol pyruvate
Ph	phenyl
PKS	polyketide synthase
Pn	pentyl
pTSA	para-toluenesulphonic acid
rpm	revolutions per minute
q	quartet
s	singlet
t	triplet
t	tertiary
TBDMS	t-butyldimethylsilyl
THF	tetrahydrofuran
THP	tetrahydropyran
TLC	thin layer chromatography
TMS	trimethylsilyl
TPP	thiamine pyrophosphate

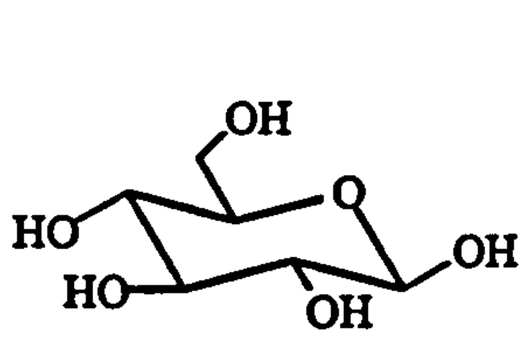
CONTENTS

Chapter 1

Introduction

1.1 Natural Products

In 1891, Emil Fischer first reported the structural elucidation of glucose (1). However, it was not until 1952, that the structure of the alkaloid morphine (2) was reported.



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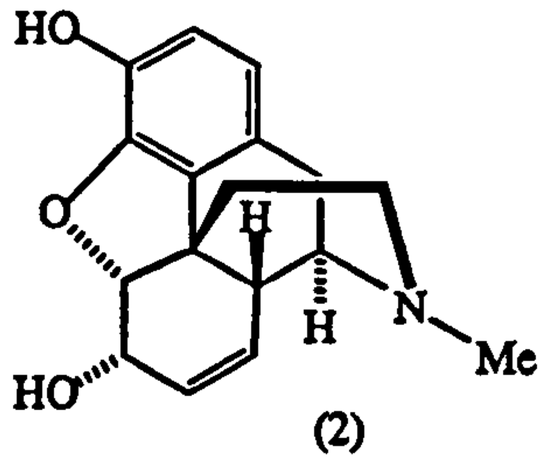

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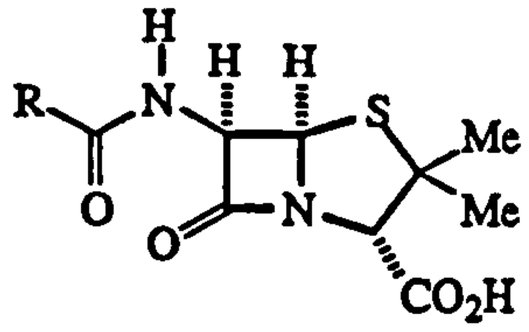


(3)

The two compounds are both natural products, yet glucose is ubiquitous and essential for the existence of life, whereas morphine is produced in just two species of poppy, *Papaver somniferum* and *P. setigerum*. Although morphine is widely used in medicine, it has no apparent function in these two plants. Similarly, penicillins (3) are produced by only a few fungal species, and despite them having no apparent use to the fungi, they have become invaluable to man as antibiotics in the last fifty years.

All organisms possess similar metabolic pathways by which they synthesise and utilise certain essential chemical species, sugars, common fatty acids, amino acids, nucleotides, and the polymers derived from them (polysaccharides, lipids, proteins, RNA, and DNA). This is primary metabolism, and these compounds are primary metabolites, which are essential for survival and well-being of the organism.[1]

Natural products such as morphine and the penicillins, which serve no apparent function for the organism, and which are found in relatively few species, are termed secondary metabolites.[2,3] For many years, these metabolites have been used as medicines, poisons, narcotics, stimulants and spices. Some examples are shown in Fig. 1.1.

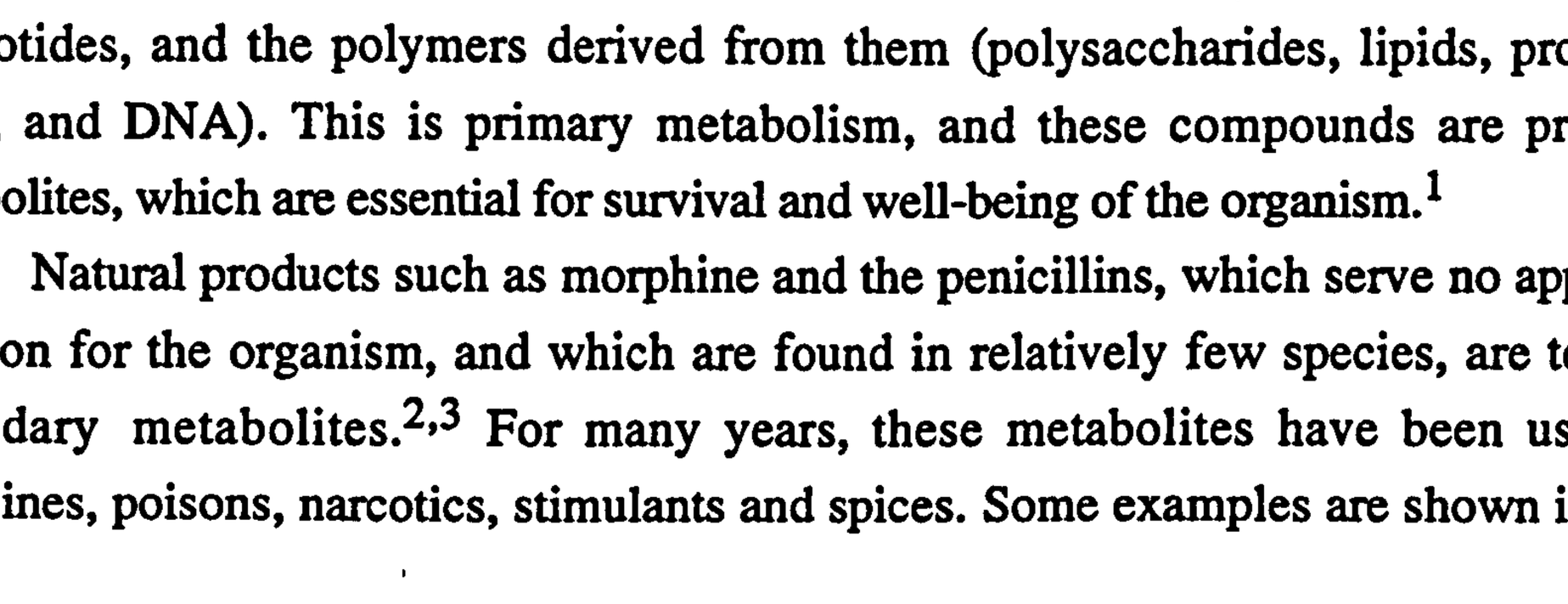
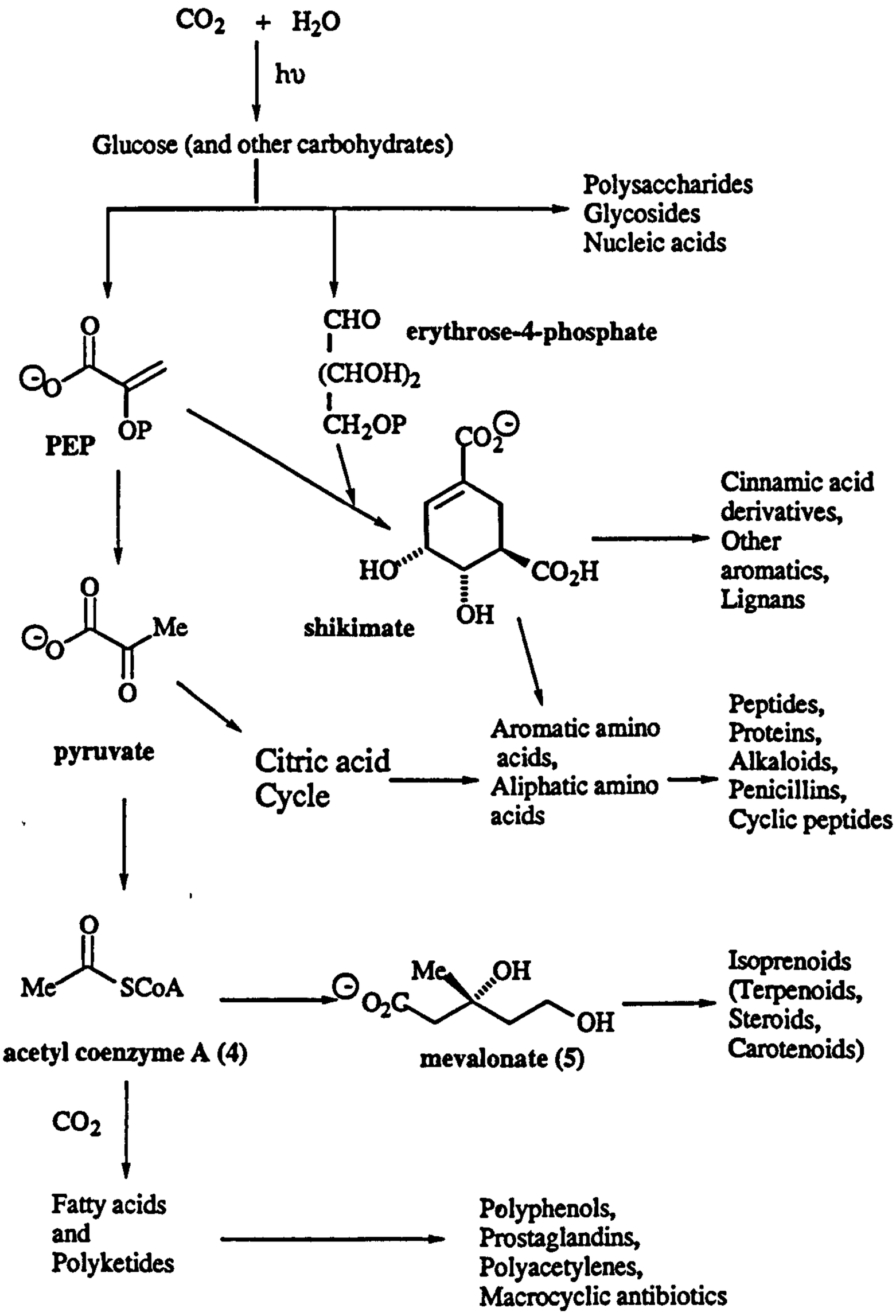


Fig. 1.1: Examples of secondary metabolites.

Primary and secondary metabolites are connected via complex metabolic pathways, as shown below in Scheme 1.1. The two areas of metabolism can be regarded as connected, since primary metabolism produces a number of small starter units: acetate, mevalonate, and shikimate. These are responsible for the biosynthesis of the secondary metabolites. Acetate, in the form of acetyl CoA (4) is the precursor for phenols and aromatics, and of polyketides and fatty acids, and their metabolites, the prostaglandins and leukatrienes. However, mevalonate (5), also derived from two molecules of acetyl CoA (4), is the precursor for the terpenes, steroids and carotenoids. Shikimate produces aromatics, whilst the amino acids are the precursors for the alkaloids, penicillins, and cephalosporins.



Scheme 1.1: Links between primary and secondary metabolism.

1.2 The elucidation of biosynthetic pathways

The isolation of natural products, in recent years has become a much simpler procedure with the advent of modern chromatographic techniques, such as HPLC. The advances in separation techniques, coupled with advances in nmr spectroscopy, infra red spectroscopy, mass spectrometry, and X-ray crystallography have facilitated the structural elucidation of a wide range of complex natural products. As a result, more time and effort can now be devoted to testing biogenetic hypotheses.

These biosynthetic investigations benefited initially from the availability of precursor molecules isotopically labelled with ^{14}C and ^{3}H, and more recently isotopically labelled with the stable isotopes ^{13}C, ^{2}H, and ^{18}O; all of which have low natural abundance, and with the exception of ^{18}O, are nmr active. ^{13}C and ^{2}H are now the most widely used isotopes in such studies. For example, if a single ^{13}C labelled precursor is incorporated into a metabolite, then the ^{13}C nmr spectrum will show enhanced signals corresponding to the carbon atoms where the ^{13}C label has been incorporated (Fig. 1.2 (b) and (c)).

Other more sensitive techniques involve the use of double labelled precursors, normally using ^{13}C together with another ^{13}C, ^{2}H, or ^{18}O. The positions at which the label is incorporated can then be determined by using ^{13}C nmr spectroscopy. If [1,2-$^{13}C_2$]acetate is incorporated intact into the metabolite, then the signals in the ^{13}C nmr spectrum corresponding to the carbons at the sites of incorporation will appear as 'triplets', in which the natural abundance signal is flanked by ^{13}C-^{13}C coupled satellites (Fig. 1.2(d)). However, if during the biosynthesis the labelled bond does not remain intact, and is broken, then only enhancement of the natural abundance singlet signals will be observed in the metabolite (Fig. 1.2(e)). These incorporation experiments therefore allow the determination of the origins of the carbon framework of natural products.

To deduce biosynthetic information on the origins of the hydrogen and oxygen atoms in a metabolite, ^{2}H and ^{18}O labelling techniques have been used to great effect. The use of deuterium labelled precursors, which can be monitored by ^{2}H nmr or by monitoring isotope induced shifts in the ^{13}C nmr spectrum, can help determine these origins. As mentioned, if a single ^{13}C labelled precursor is incorporated into a metabolite, then the ^{13}C nmr spectrum will show enhanced signals corresponding to the carbon atoms, where the ^{13}C label has been incorporated. Analogous results are obtained with the use of ^{2}H labelled precursors, and the resulting δ_H nmr spectra.

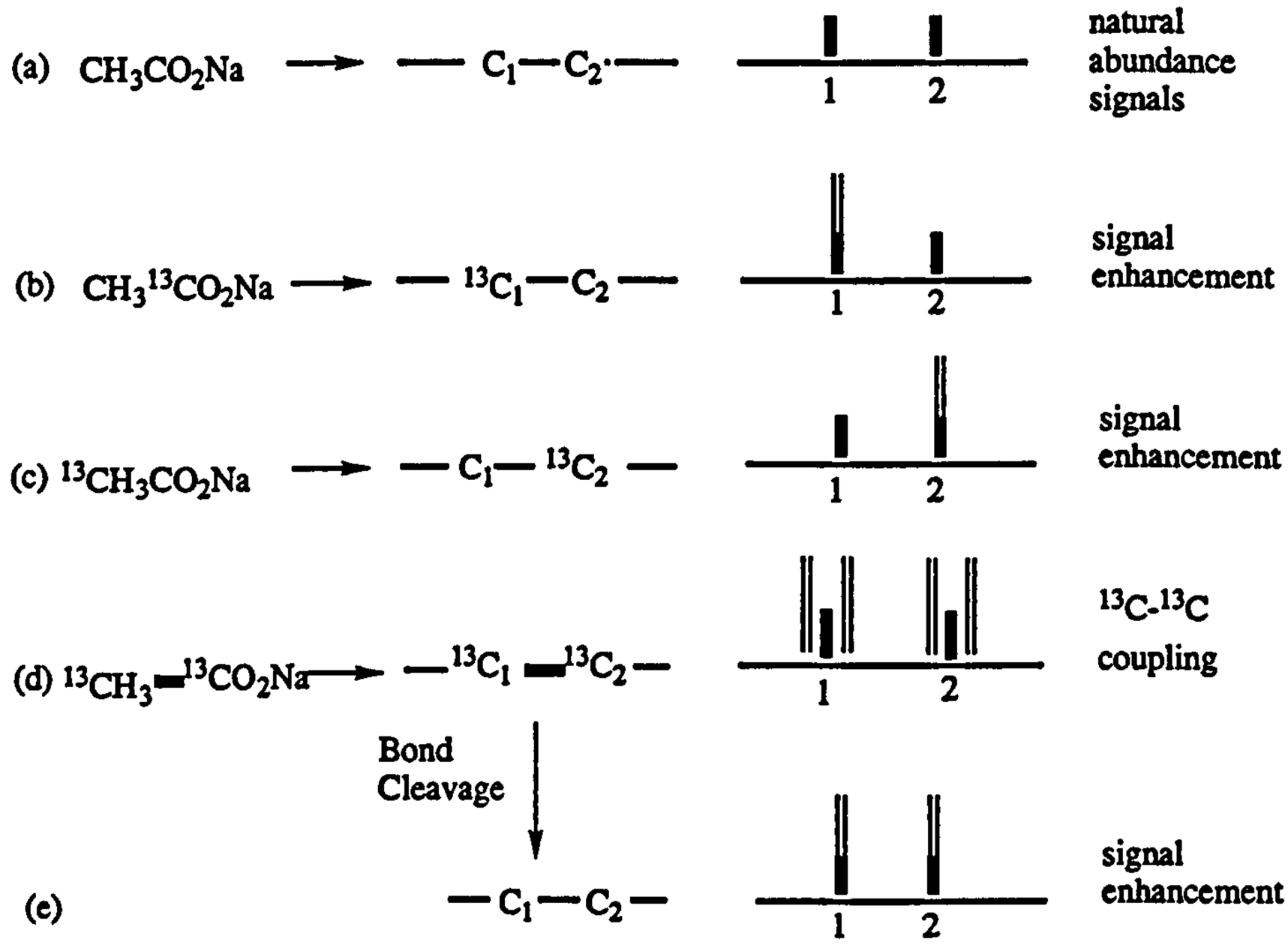


Fig. 1.2: The use of singly and doubly ^{13}C labelled precursors in biosynthetic studies, using ^{13}C nmr spectroscopy.

However, ^{2}H nmr spectroscopy has its limitations. It often produces poorly resolved spectra, due to its quadrupole nucleus (spin=1), a low gyromagnetic constant, and a small chemical shift range, compared with ^{13}C. The advantage of ^{2}H nmr spectroscopy is that deuterium has a very low natural abundance (0.012% for ^{2}H, compared with 1.1% for ^{13}C), hence, it is particularly suitable for studying incorporation of assembly intermediates into secondary metabolites, where low incorporation of a labelled precursor into the metabolite is often found. Low incorporation of a labelled precursor into a metabolite is also a reason why nmr spectroscopy is used in preference to mass spectrometry.

The deuterium can be located either one (α) or two (β) bonds away from the ^{13}C nucleus. Hence, incorporation of [2-^{13}C, $^{2}H_3$]acetate leads to a multiplet in the ^{13}C nmr spectrum, due to $^{13}C/^{2}H$ coupling. This coupling has the effect of CD appearing as a triplet, CD_2 as a quintet, and CD_3 as a septet. Also, the presence of each deuterium also produces an upfield α-isotope shift of the signal by approximately 0.03ppm. (Fig. 1.3(a)). This can lead to complex spectra, but can be overcome using deuterium decoupling, or by using deuterium placed β to the ^{13}C nucleus. Here, the $^{13}C/^{2}H$ coupling is too small to be resolved, and the signals appear as upfield singlets with a β isotope shift of the ^{13}C nmr spectrum, typically 0.01 to 0.1ppm. Incorporation of [1-^{13}C, $^{2}H_3$]acetate illustrates this point (Fig. 1.3(b)).

Incorporation of [1-^{13}C, $^{18}O_2$]acetate into a metabolite enables the biosynthetic origins of acetate derived oxygen to be determined. This leads to an upfield α-isotope shift of 0.01 to 0.05ppm (Fig. 1.3 (c)). A similar effect can be observed when organisms

are grown in an $^{18}O_2$ enriched atmosphere. Atmospheric derived oxygen atoms may be labelled within the metabolite, and as a result, show a small ^{18}O-isotope shift in the ^{13}C nmr spectrum (Fig. 1.3(d)).

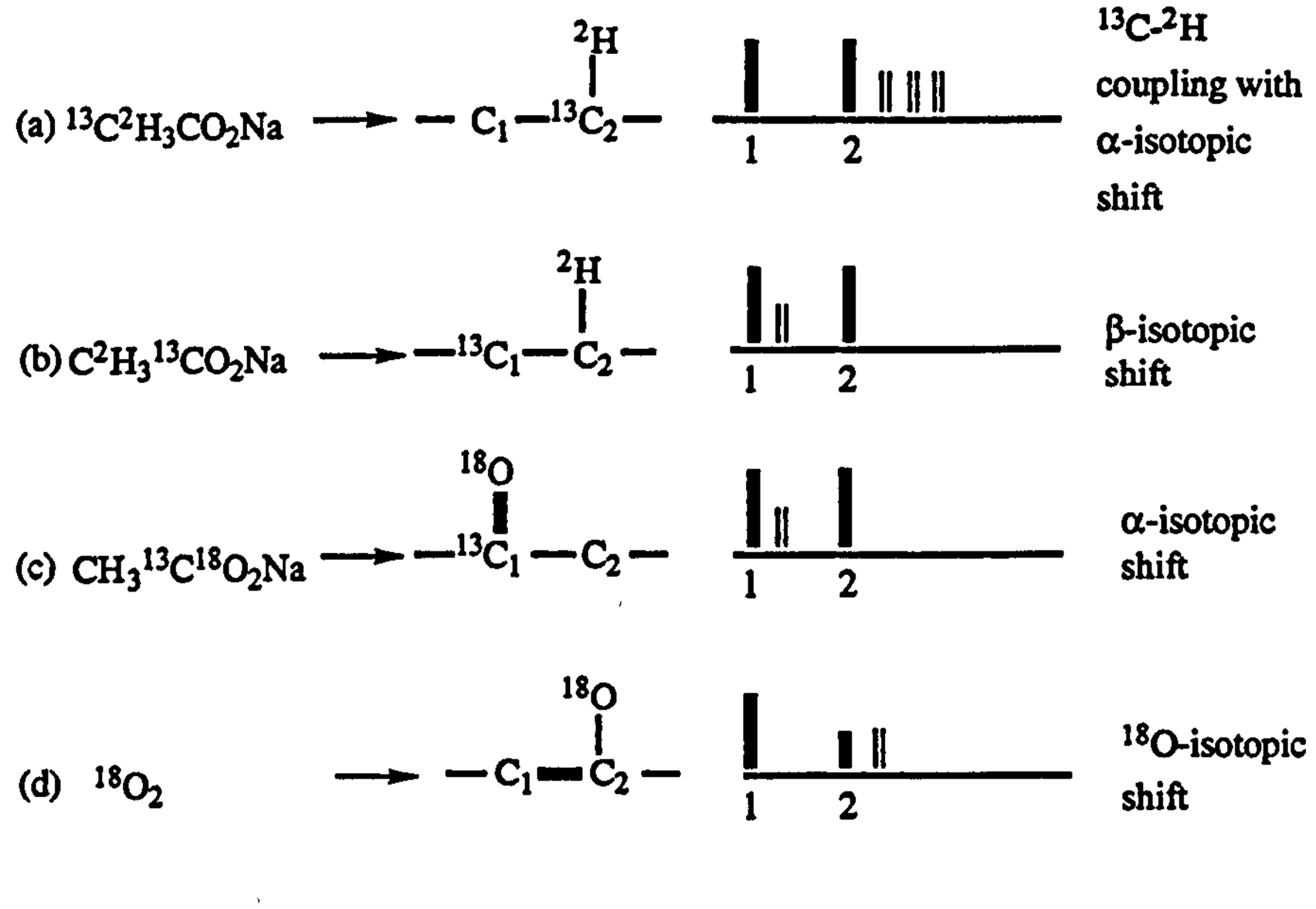


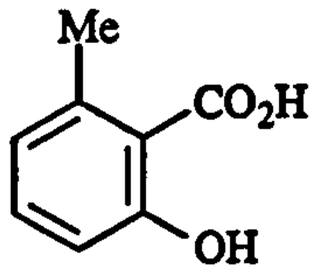
Fig. 1.3: Predicted patterns in ^{13}C nmr spectra of ^{2}H and ^{18}O labelled metabolites.

Specific examples of the use of ^{13}C, ^{2}H, and ^{18}O, in the elucidation of the biosynthetic pathways to polyketides, will be discussed in section 1.3.

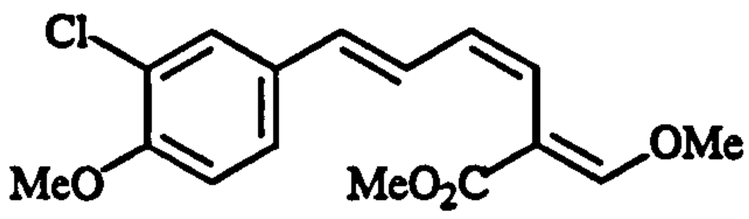
1.3 Polyketide Biosynthesis

The work described in this thesis was aimed at elucidating the biosynthesis of pseudomonic acid, a polyketide derived antibiotic, produced by *Pseudomonas fluorescens*. Polyketides comprise of a wide range of naturally occurring products, and have been isolated by fungi, bacteria, insects, marine organisms, and higher plants.[4,5]

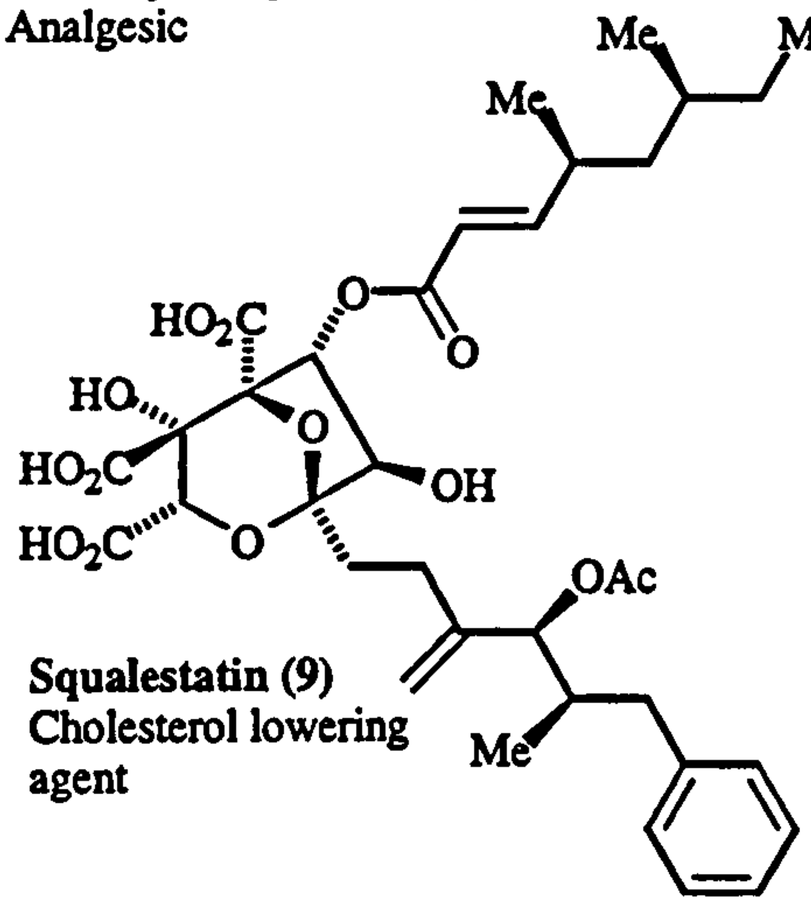
The polyketide biosynthetic pathway is one of the most widespread in nature. A wide range of biologically active natural products are polyketide derived, including the antibiotics 6-methylsalicylic acid (6-MSA) (6), tetracycline, and erythromycin B (7), the antifungal agents griseofulvin and the strobilurins (8), the cholesterol lowering agents lovastatin and squalestatin (9), and the immunosuppressive agents rapamycin (10) and FK506; some of which are shown in Fig. 1.4.



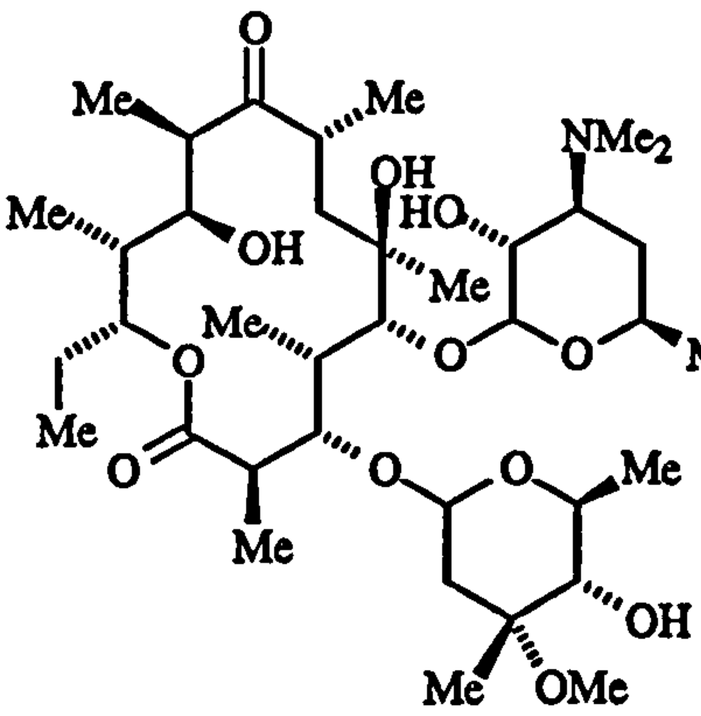
6-Methylsalicylic acid (6)
Analgesic



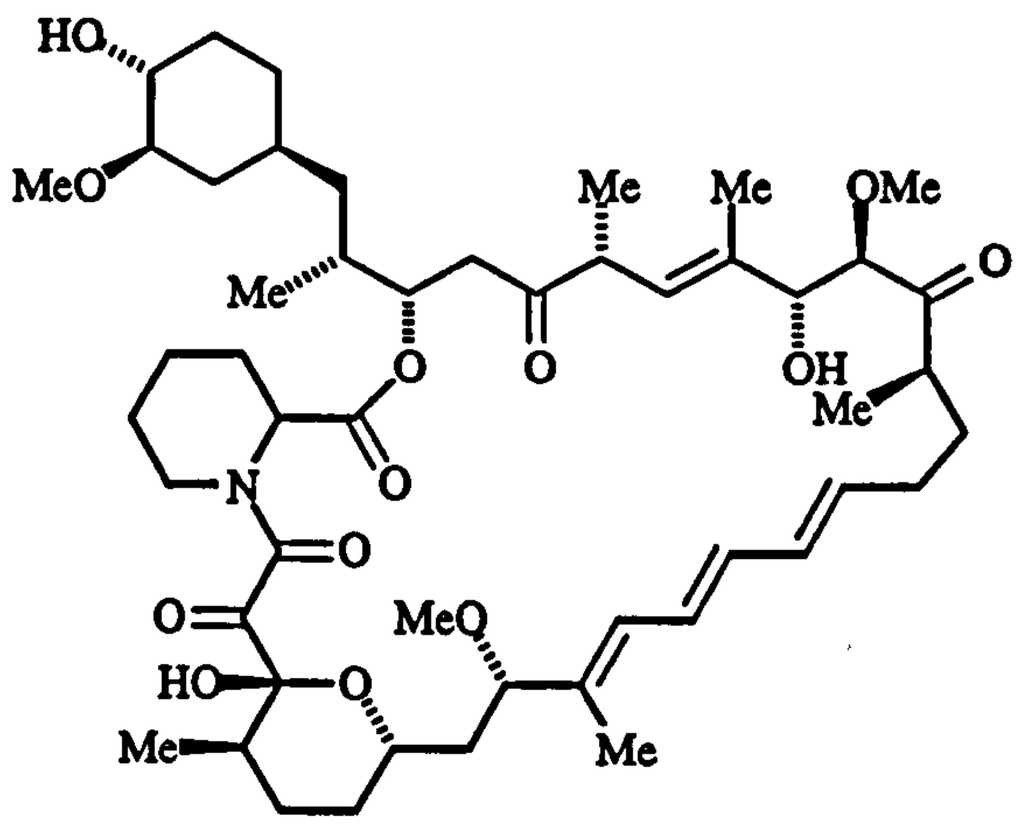
Strobilurins (8)
Antifungal



Squalestatin (9)
Cholesterol lowering agent



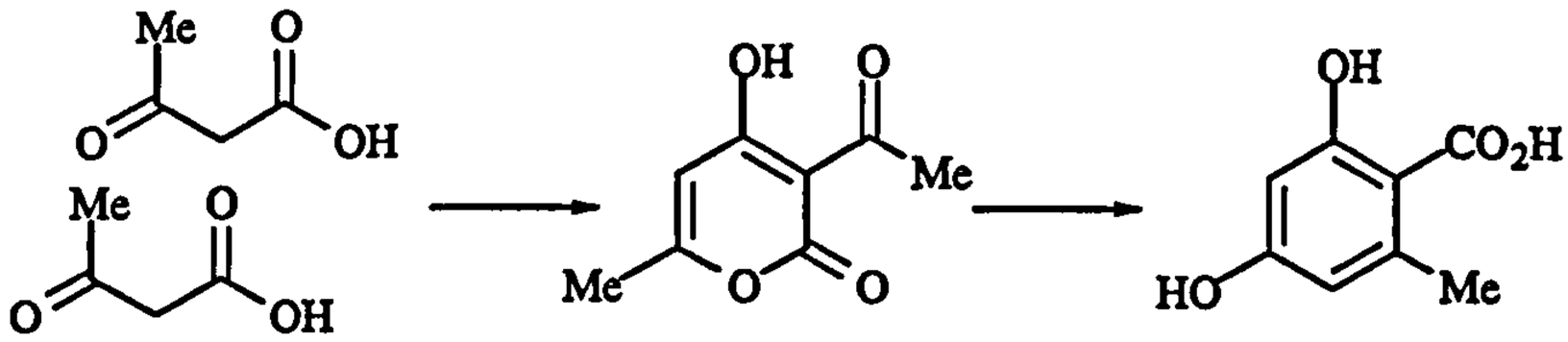
Erythromycin B (7)
Antibiotic



Rapamycin (10)
Immunosuppressant

Fig. 1.4: Examples of the structural diversity exhibited by polyketide derived metabolites.

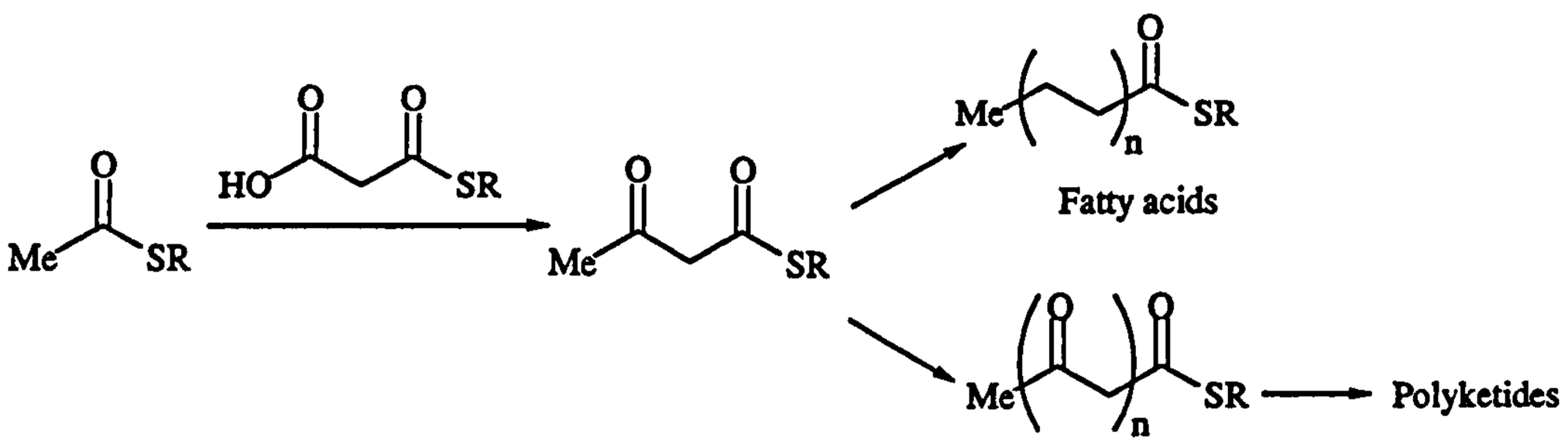
Despite their structural diversity, all of the polyketides are related by their common biosynthetic origins. The biosynthetic pathway has been studied for many years. In 1907, Collie put forward a series of biosynthetic hypotheses,[6] based on the observation that many natural products contained within them the repetitious $[CH_2\text{-}CO]_n$ unit. To verify these hypotheses he described experiments which showed that orsellinic acid (11) could be formed from two molecules of acetoacetic acid (12), via dehydroacetic acid (13) as outlined in Scheme 1.2.[6]



(12) (13) (11)

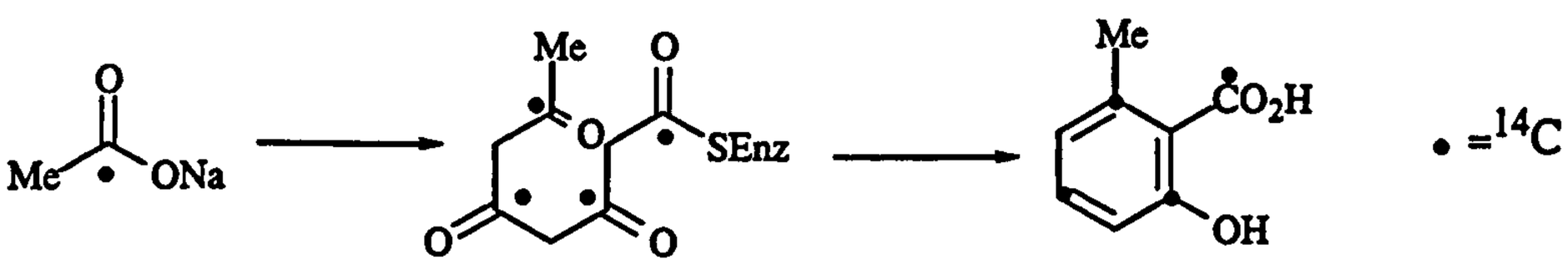
Scheme 1.2: The biosynthesis of orsellinic acid as proposed by Collie.

These ideas were largely ignored, until 1953, when Birch proposed that the structures of many secondary metabolites could be correlated with the repeating [CH_2-CO] units, that would result from a biogenesis via acetate.[7] Thus orsellinic acid (12) could be thought of as arising through the poly-β-ketoacyl CoA derivative, derived by a linear combination of acetate units. This invoked a hypothesis involving retention of the β-keto functions in the linear chain, resulting from sequential acetate-malonate condensations, as shown in Scheme 1.3.



Scheme 1.3: Acetate-malonate condensations leading to fatty acids and polyketides.

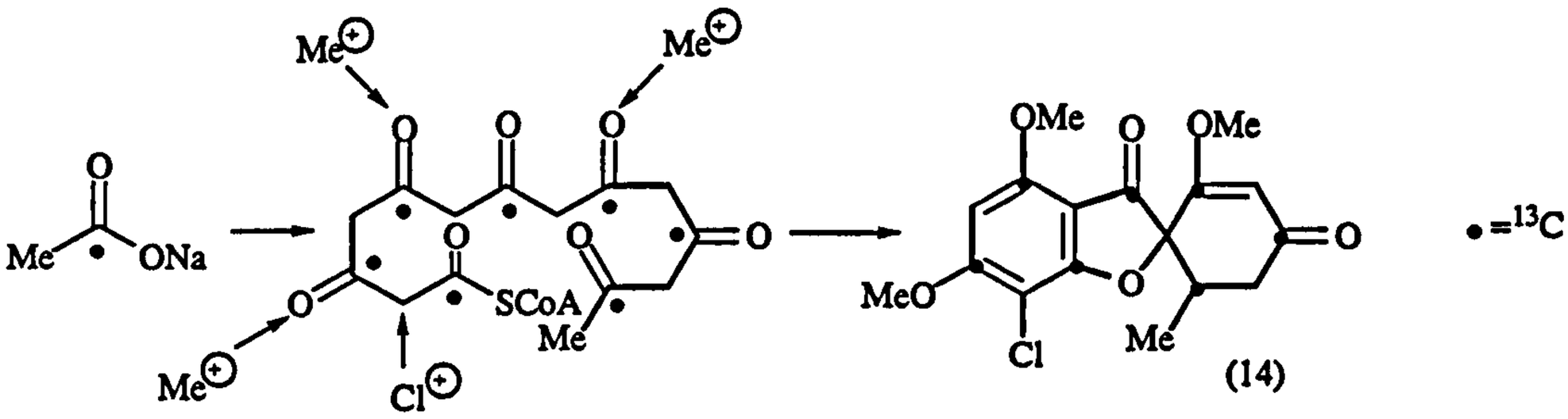
Birch provided the first experimental evidence to support his hypothesis, showing that ^{14}C-acetate was incorporated into 6-MSA (6) in *Penicillium griseofulvin*, with the correct alternate labelling pattern, as outlined in Scheme 1.4.[8]



(6)

Scheme 1.4: The biosynthesis of 6-MSA.

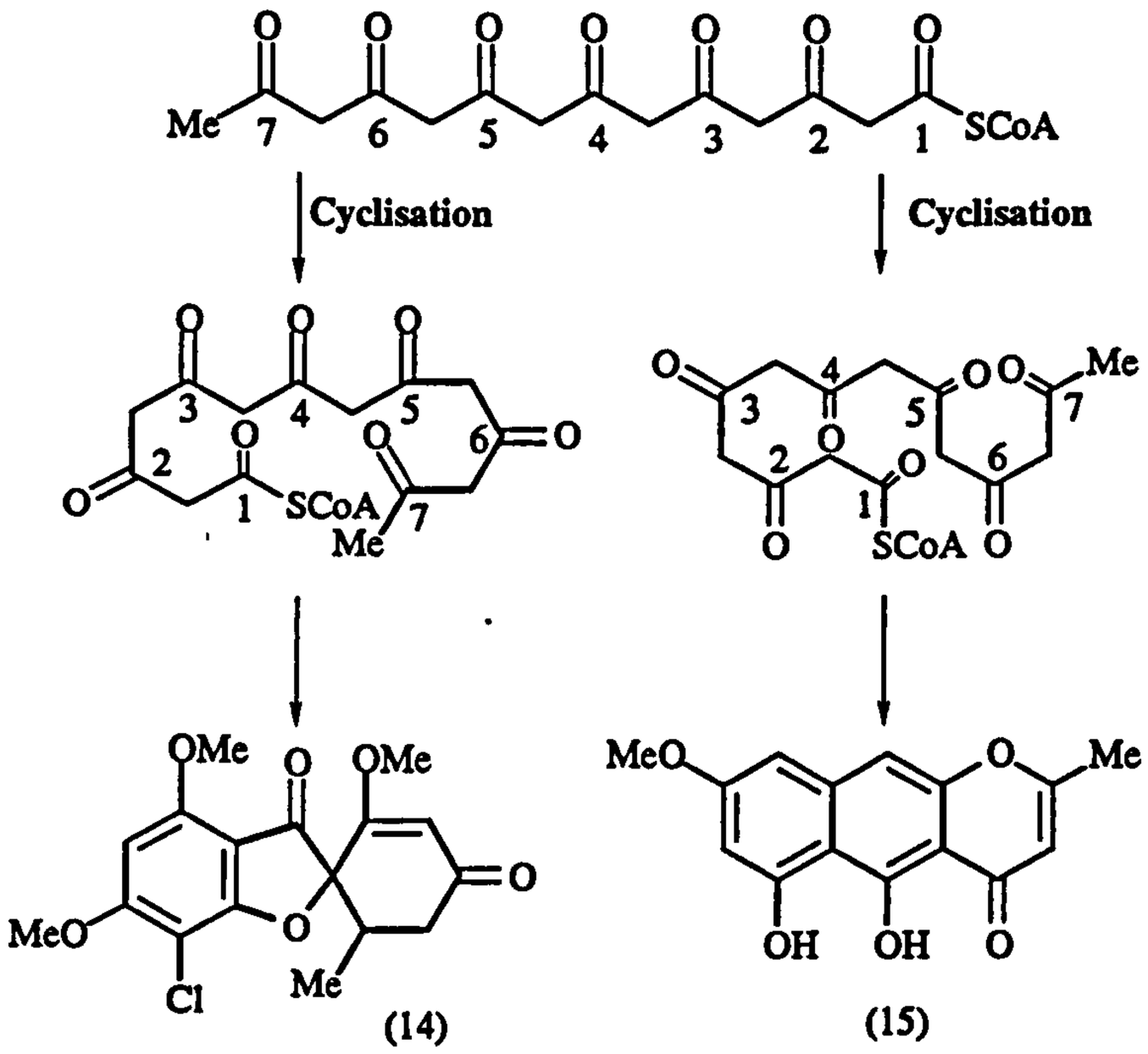
Further work showed that other metabolites were of polyketide origin, including griseofulvin (14) an antifungal isolated from *Penicillium griseofulvin*.[9] This was shown to be a heptaketide, with the methyl groups, originating from S-adenosylmethionine.



Scheme 1.5: The biosynthesis of griseofulvin.

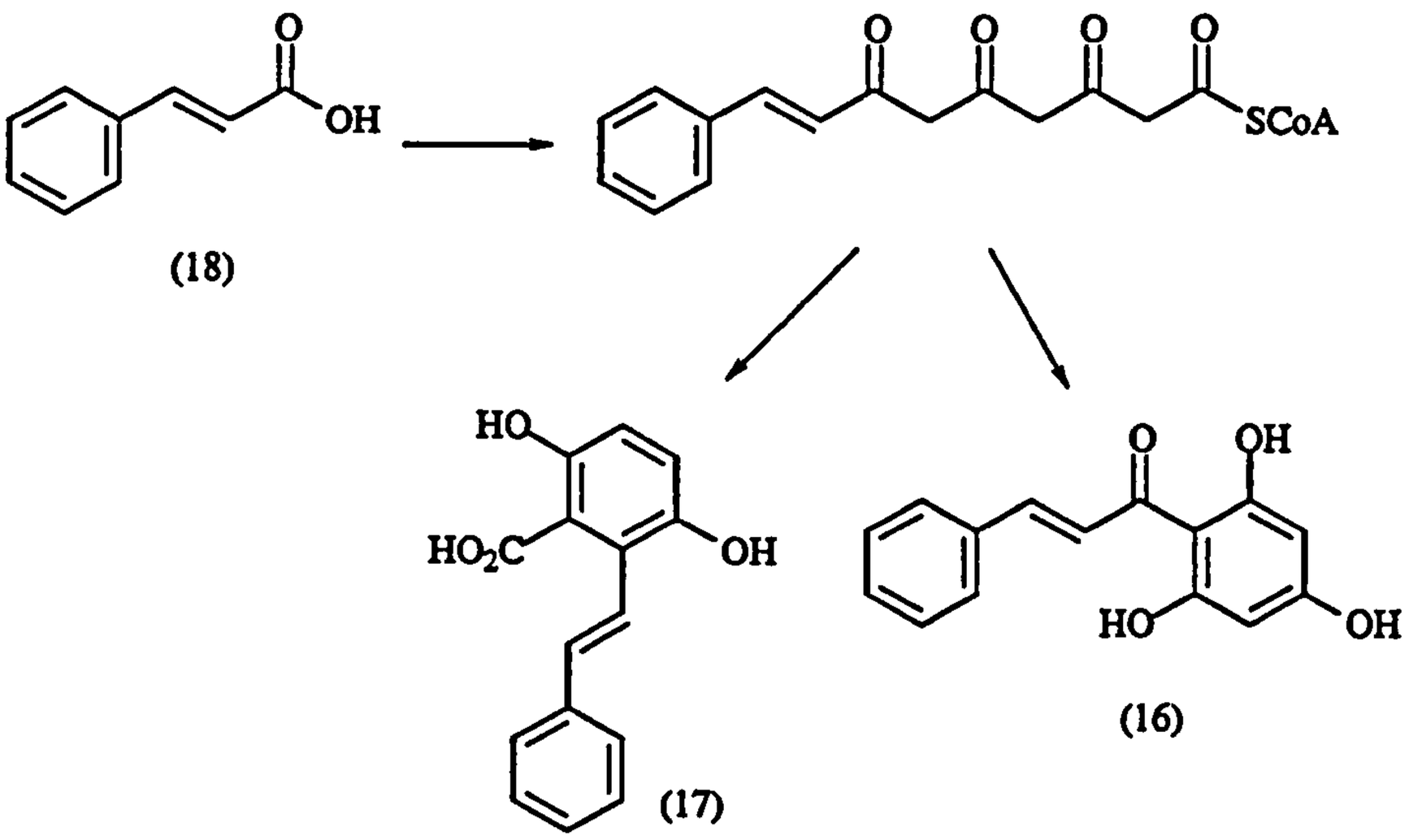
Birch showed that many polyketide metabolites share a common biosynthetic origin through the folding of poly-β-keto chains, derived from sequential acetate-malonate condensations. Hence, polyketide biosynthesis was found to be analogous to fatty acid biosynthesis, in terms of the starter unit acetate condensing with a malonate unit.

To explain the structural diversity and various oxidation states exhibited by the polyketides, it is important to realise that the poly-β-keto chains can undergo cyclisations, aromatisations, and reductive modifications in any order, starting from a different conformation. Consider a poly-β-keto heptaketide, it may fold in many ways, thus giving rise to a whole range of metabolites. These products can exhibit different oxidation levels, *e.g.* griseofulvin and rubrofusarin (15).



Scheme 1.6: Modifications of poly-β-keto heptaketide chains, giving griseofulvin and rubrofusarin.

Birch introduced the idea of the sequential condensation of acetate and malonate, prior to chain modifications, in order to explain the formation of many aromatic polyketides. The hypothesis was modified to accommodate the introduction of structural diversity, by the use of different chain starting or extending units.[10] For example, chalcone (16) and stilbene (17) biosyntheses were shown to originate from a *trans* -cinnamic acid starter unit (18), as shown in Scheme 1.7.[11]

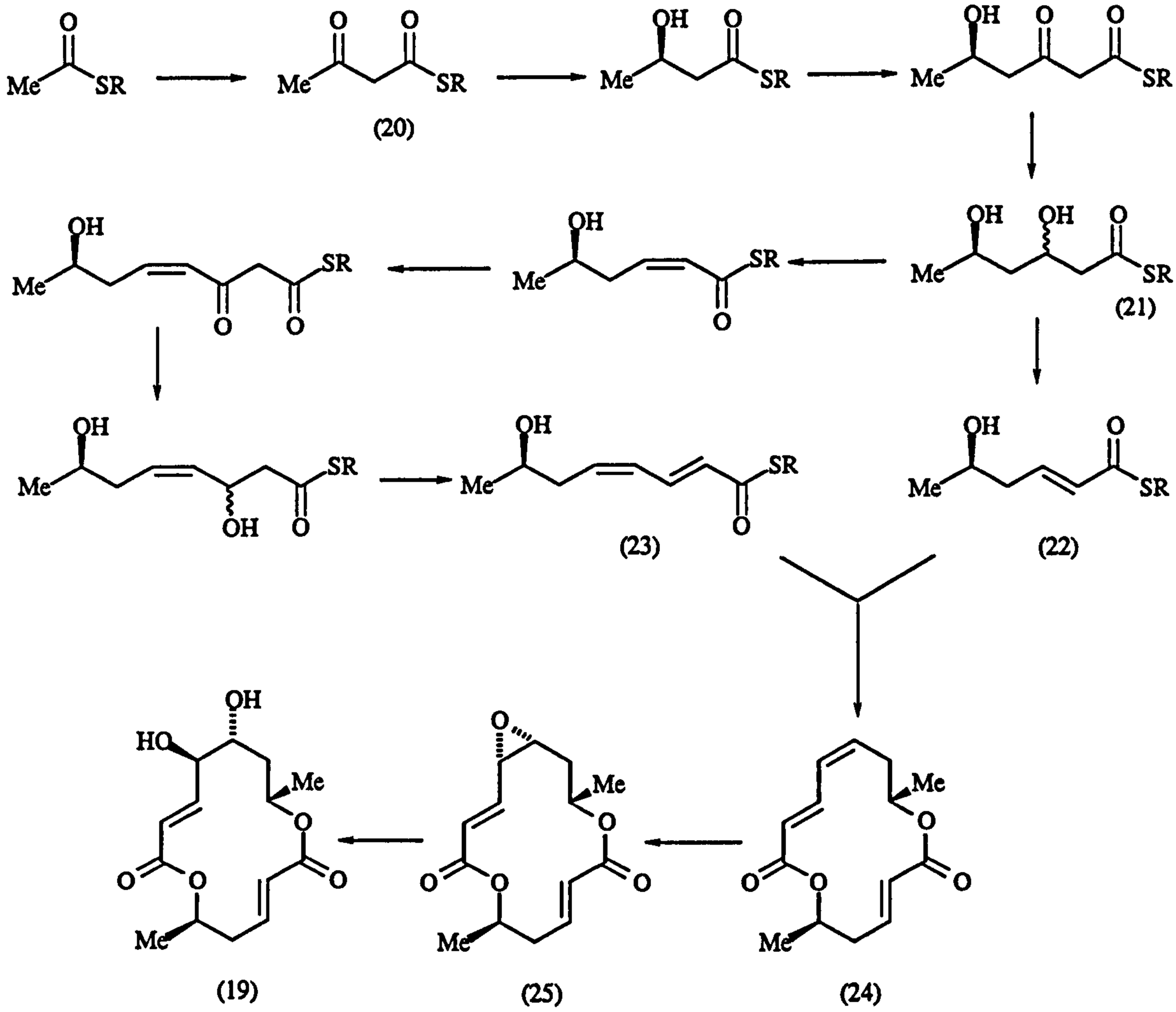


(18) (17) (16)

Scheme 1.7: Cinnamic acid as a chain starter unit in chalcone and stilbene biosynthesis.

Further work has shown that many non-aromatic natural products belong to the polyketide class. For example, the macrodiolide colletodiol (19), is a simple non-aromatic polyketide. Isotopic labelling studies by MacMillan and Lunnon,[12] and by Simpson and Stevenson,[13] accounted for the origins of all the carbons, oxygens, and hydrogens in colletodiol. These observations led to a proposed biosynthetic pathway to colletodiol, starting with an acetate and malonate condensation to give enzyme bound acetoacetate (20) (Scheme 1.8). Subsequent reduction, condensation, and reduction would produce the intermediate diol (21). Dehydration of the diol may occur to either give the C_6 hydroxy acid triketide (22) with a *trans* double bond, or following addition of another C_2 unit may afford the C_8 hydroxy acid tetraketide (23). Coupling of a C_6 hydroxy acid (22) and a C_8 hydroxy acid (23) would lead to colletotriene (24), which on oxidation with atmospheric oxygen would lead to colletoepoxide (25). Hydrolytic ring opening of the epoxide with water from the medium leads to colletodiol.

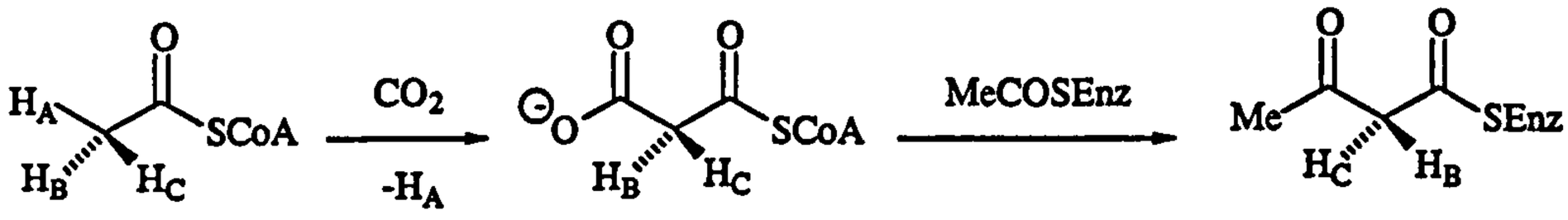
Intact incorporation of both deuterated colletotriene and deuterated colletoepoxide into colletodiol have confirmed the later stages of the proposed biosynthetic pathway.[14,15]



Scheme 1.8: The proposed biosynthesis of colletodiol.

Although, it was originally proposed that extended poly-β-keto chains were formed, and then modified in later steps,[7] studies on compounds such as colletodiol led to the idea of a 'processive' mode of polyketide biosynthesis, in which the intermediates are reduced and modified during chain elongation, rather than afterwards. As a result, it is now accepted that there is substantial similarity between the assembly of polyketides by the polyketide synthases (PKSs), and the assembly of fatty acids by fatty acid synthases (FASs).

The construction of fatty acids is accomplished within a multienzyme complex, known as fatty acid synthase (FAS). Fatty acid biosynthesis begins with the starter unit acetyl CoA (Scheme 1.9). Acetyl CoA is carboxylated to malonyl CoA, using acetyl CoA carboxylase, with retention of configuration (Scheme 1.9).[16,17] Acetyl CoA is then transfered to the active site of the thiol of the condensing enzyme, ketoacyl synthase (KS), whilst malonyl CoA is transferred to the acyl carrier protein (ACP) by malonyl transferase (MT). Condensation of enzyme bound acetate with enzyme bound malonate produces enzyme bound acetoacetate, with simultaneous loss of carbon dioxide, with inversion of configuration (Scheme 1.9).[16,17]



Scheme 1.9: Carboxylation of acetyl CoA, showing retention of configuration, and condensation of acetate and malonate to produce enzyme bound acetoacetate, with inversion of configuration.

Stereospecific reduction by ketoreductase (KR) produces the (3*R*)-β-hydroxy intermediate, and *syn* elimination by dehydratase (DH) follows to give the α,β-unsaturated intermediate.[18] Further reduction by enoyl reductase (ER) produces the fully saturated moiety, before further chain extension takes place.

Polyketides are derived from highly functionalised carbon chains whose assemblies are controlled by multifunctional enzyme complexes, called polyketide synthases (PKSs). Like the FASs, PKSs catalyse a repetitious sequence of decarboxylative condensation reactions between acyl thioesters and malonate chain extender units. Each condensation is followed by a varying cycle of reductive modifications: ketoreduction, dehydration, and enoyl reduction. In contrast to fatty acid biosynthesis, where the full cycle of reductive modifications normally follows each condensation, the PKSs can use this sequence in a controlled fashion to assemble polyketides of great structural diversity, and of varying oxidation states.

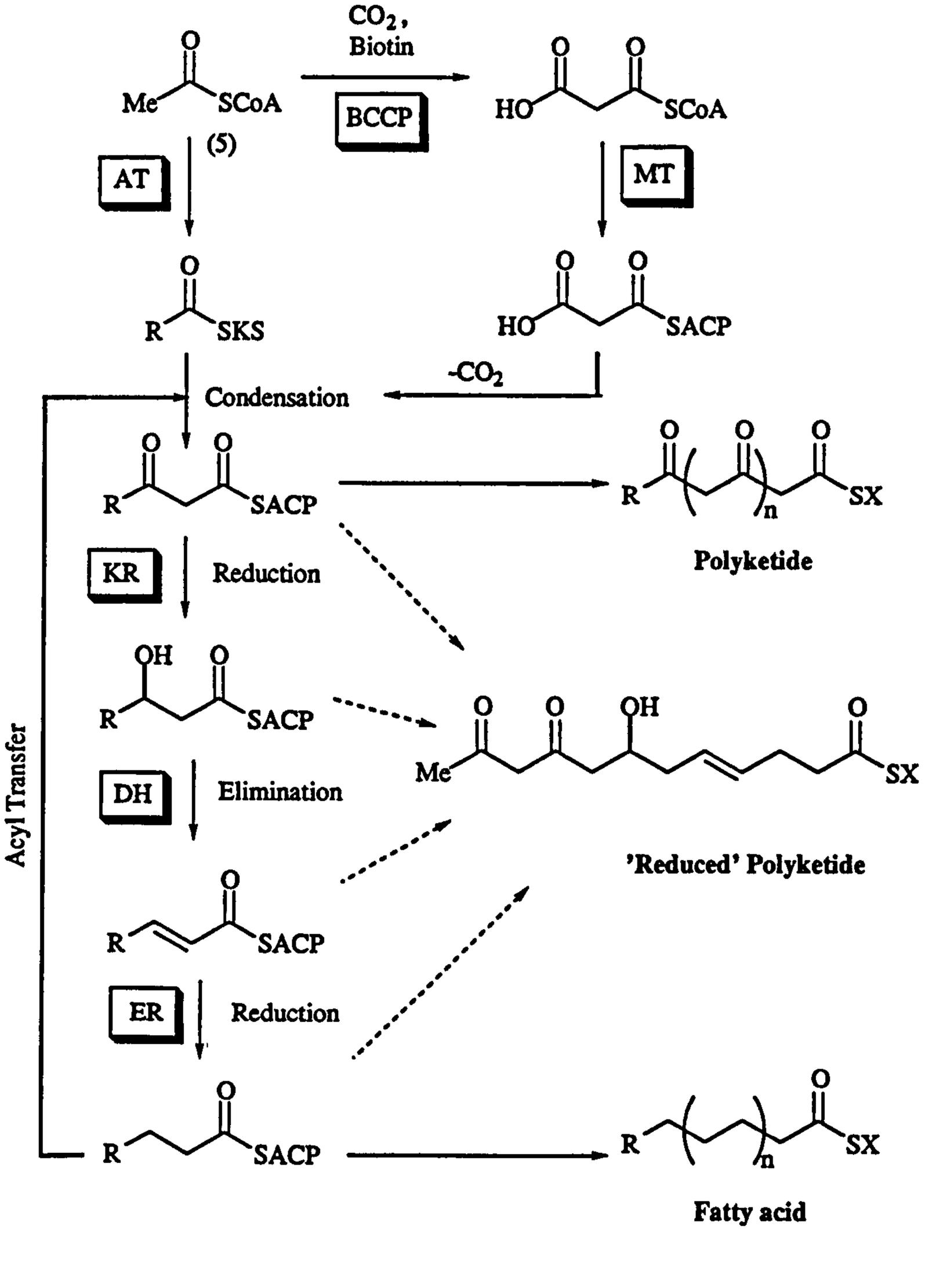
As shown in Scheme 1.9, the reduction sequence can be completely omitted, thus producing the classical polyketide, with a carbonyl group on every alternate carbon. However, reductive modifications can be used fully or partially after each condensation to yield highly functionalised intermediates. This is known as the processive mode of assembly of polyketides.[19]

It is important to note several differences between fatty acid and polyketide biosynthesis:

(i) In fatty acid biosynthesis, the acyl thioesters normally involved in the condensations are acetyl CoA and malonyl CoA. However, PKSs can use a variety of chain starter units, including acetate, propionate, benzoate, cinnamate and some amino acids.

(ii) Different extender units maybe employed in polyketide biosynthesis; including malonate, methylmalonate, and ethylmalonate. Branching methyl groups may also be introduced by S-adenosylmethionine.

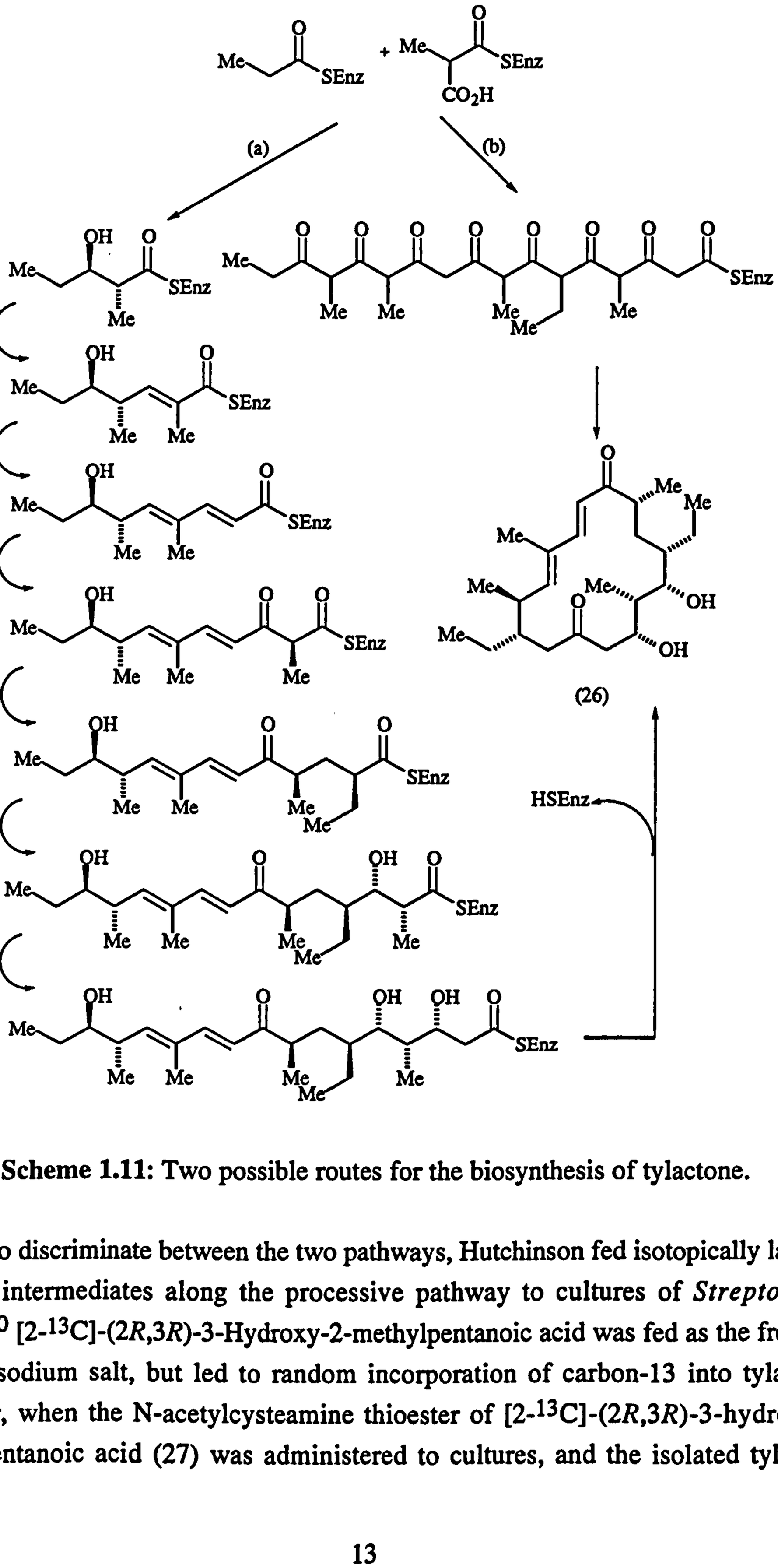
(iii) PKSs can use all, some, or none of the post-condensation reductive modifications, thus producing a wide range of metabolites containing β-keto, β-hydroxy, α,β-unsaturated, or fully saturated units. Formation of a polyketide therefore involves condensation of acetate (or another starter unit) with the appropriate number of malonate units, modification of the poly-β-ketoacyl CoA where required, and then the release of a stable form of the product.



Key:	AT:	Acyl transferase	BCCP:	Biotin carboxylate carrier protein
	KS:	Ketosynthase		
	KR:	Ketoreductase	ACP:	Acyl carrier protein
	DH:	Dehydratase	MT:	Malonyl transferase
	ER:	Enoyl reductase	CoA:	Coenzyme A

Scheme 1.10: The assembly of fatty acids, polyketides, and 'reduced' polyketides.

Scheme 1.11 shows the two possible routes for the biosynthesis of tylactone (26): the processive mode of assembly (Path (a)), and the classical polyketide route (Path (b)).

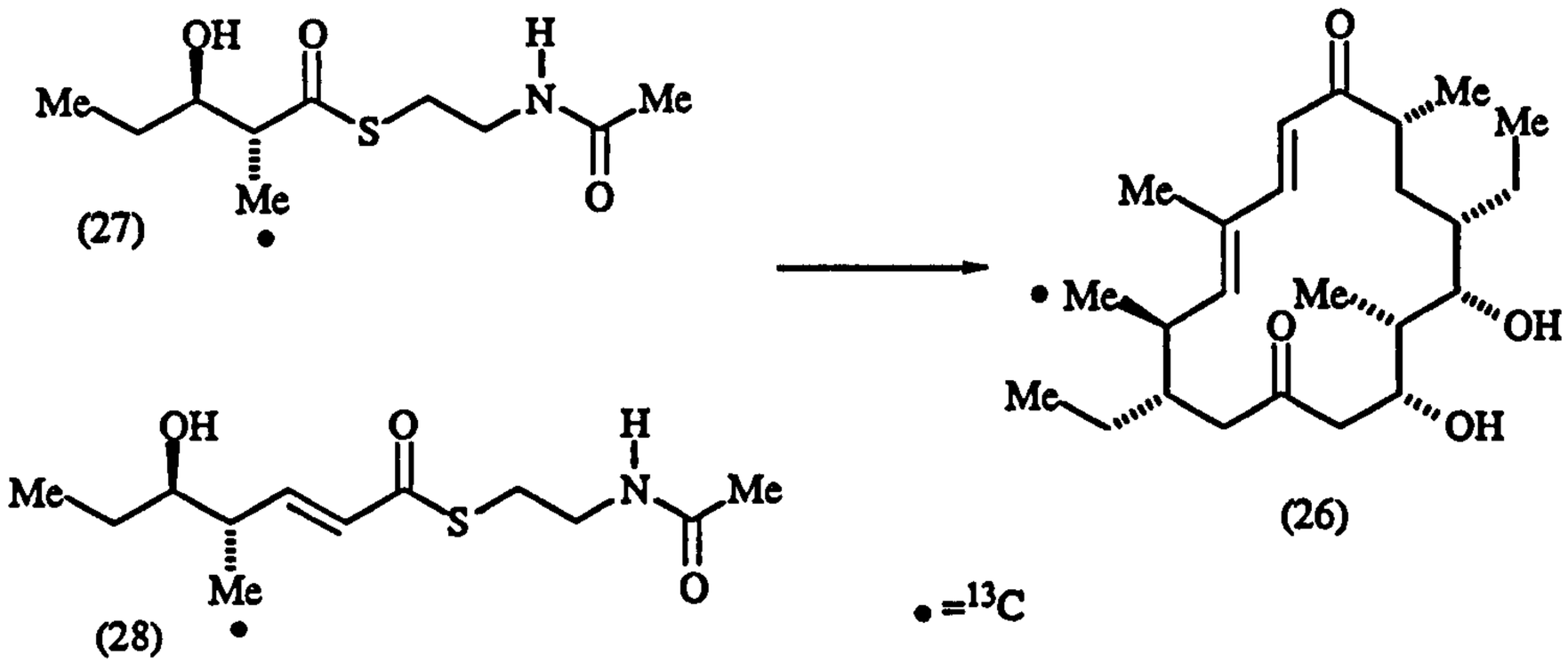


Scheme 1.11: Two possible routes for the biosynthesis of tylactone.

To discriminate between the two pathways, Hutchinson fed isotopically labelled putative intermediates along the processive pathway to cultures of *Streptomyces fradiae*.[20] [2-^{13}C]-(2*R*,3*R*)-3-Hydroxy-2-methylpentanoic acid was fed as the free acid and the sodium salt, but led to random incorporation of carbon-13 into tylactone. However, when the N-acetylcysteamine thioester of [2-^{13}C]-(2*R*,3*R*)-3-hydroxy-2-methylpentanoic acid (27) was administered to cultures, and the isolated tylactone

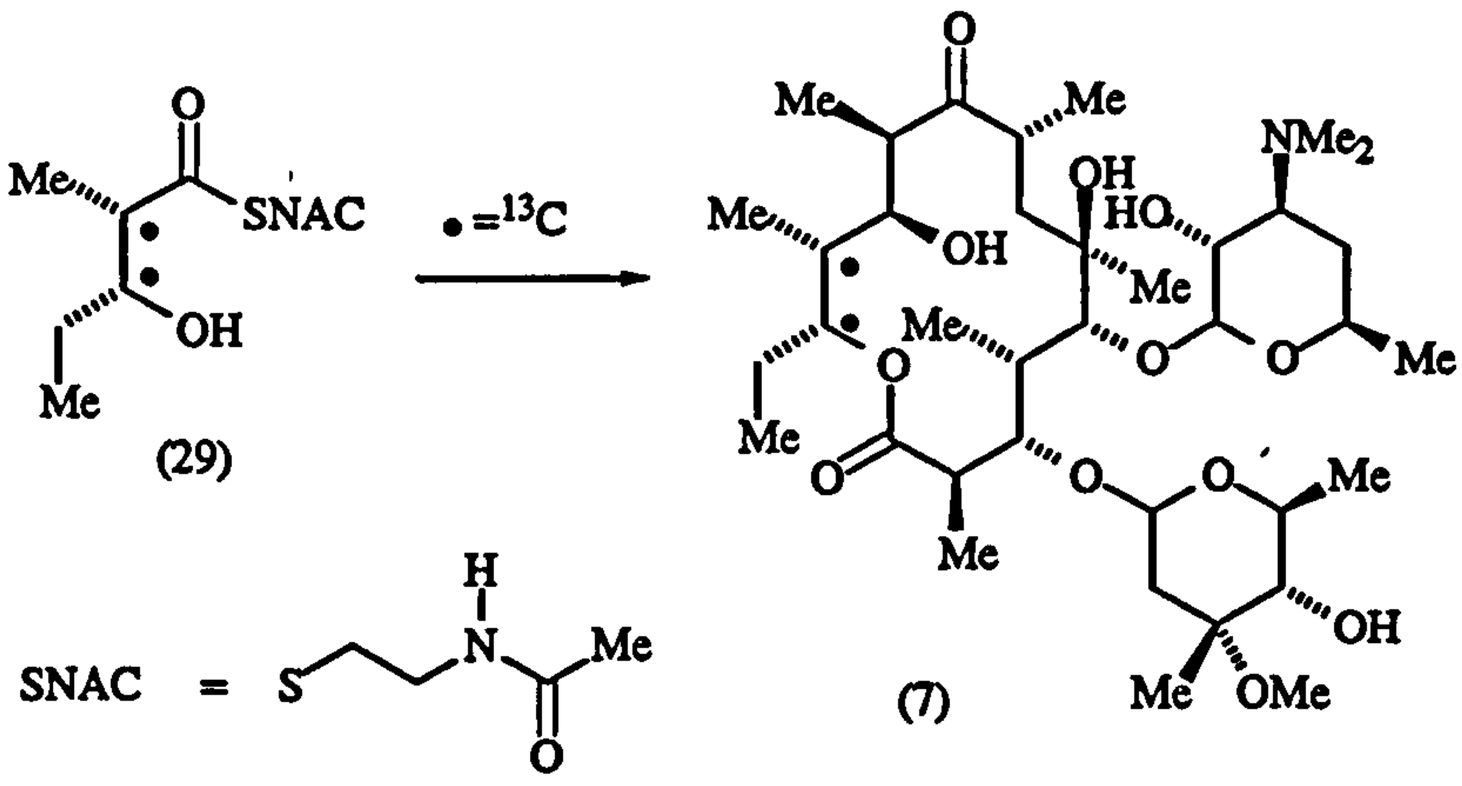
analysed by ^{13}C nmr spectroscopy, specific incorporation of the label into C-18 was observed, as predicted by the processive mode of assembly (Scheme 1.12).[20] The use of N-acetylcysteamine thioesters in feeding studies will be discussed in section 2.4.

As predicted from the processive mode of assembly feeding the N-acetylcysteamine thioester of [4-^{13}C]-(2*E*,4*R*,5*R*)-2,4-dimethyl-5-hydroxyhept-2-enoic acid (28) to cultures of *Streptomyces fradiae* gave specific incorporation of carbon-13 into C-18 of tylactone.[20]



Scheme 1.12: Incorporation of chain assembly intermediates into tylactone, produced by *S. fradiae*.

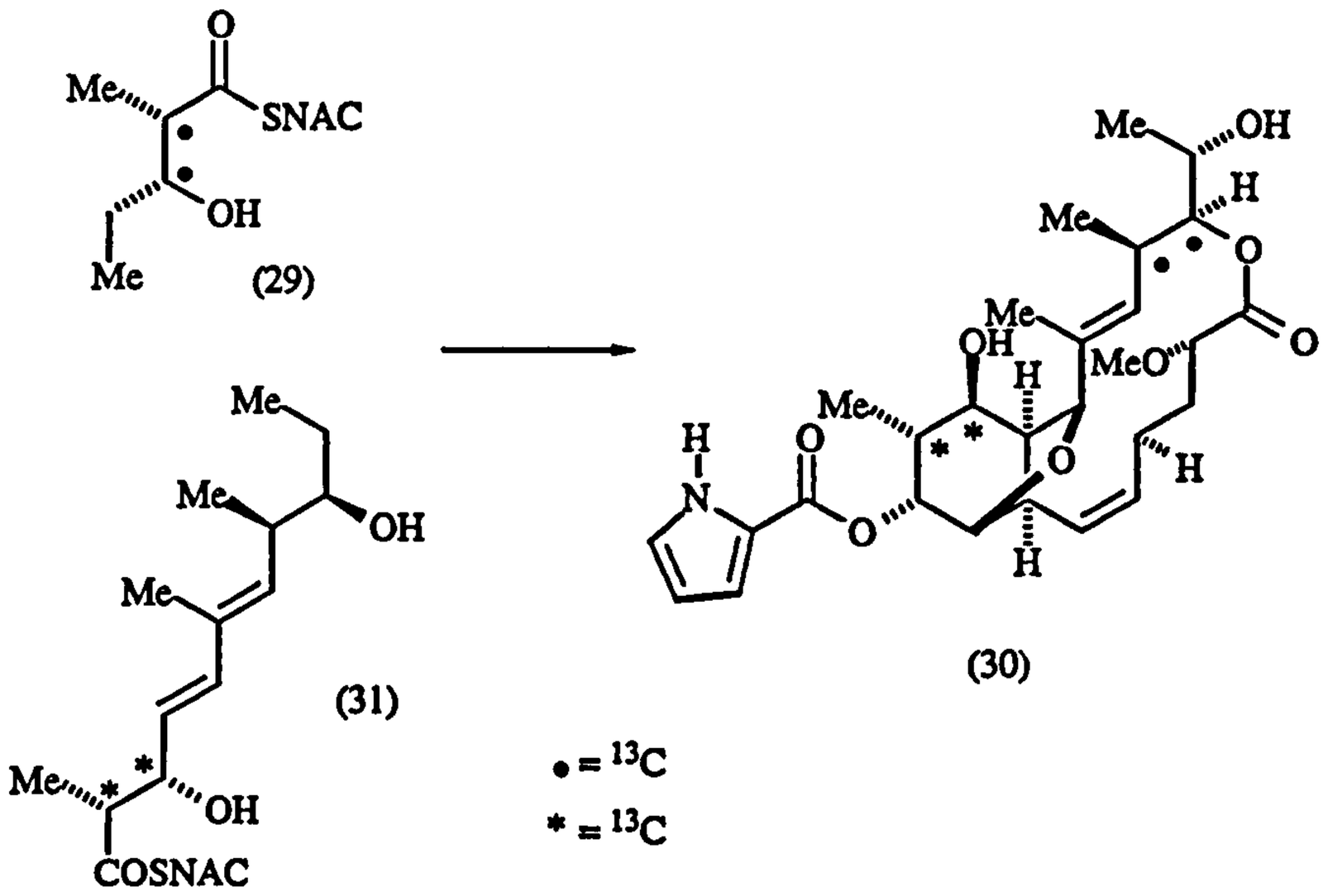
Cane provided evidence that a processive mode of assembly was operating in the biosynthesis of erythromycin B.[21] The N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-(2*S*,3*R*)-3-hydroxy-2-methylpentanoic acid (29) was fed to *Streptomyces erythreus*, isolation of erythromycin B and analysis of its ^{13}C nmr spectrum revealed specific incorporation of carbon-13 into C-12 and C-13 (Scheme 1.13).[21]



Scheme 1.13: Incorporation of chain assembly intermediates into erythromycin B, produced by *S. erythreus*.

Subsequently, Cane reported further success with the intact incorporation of isotopically labelled putative intermediates to confirm a processive mode of assembly of

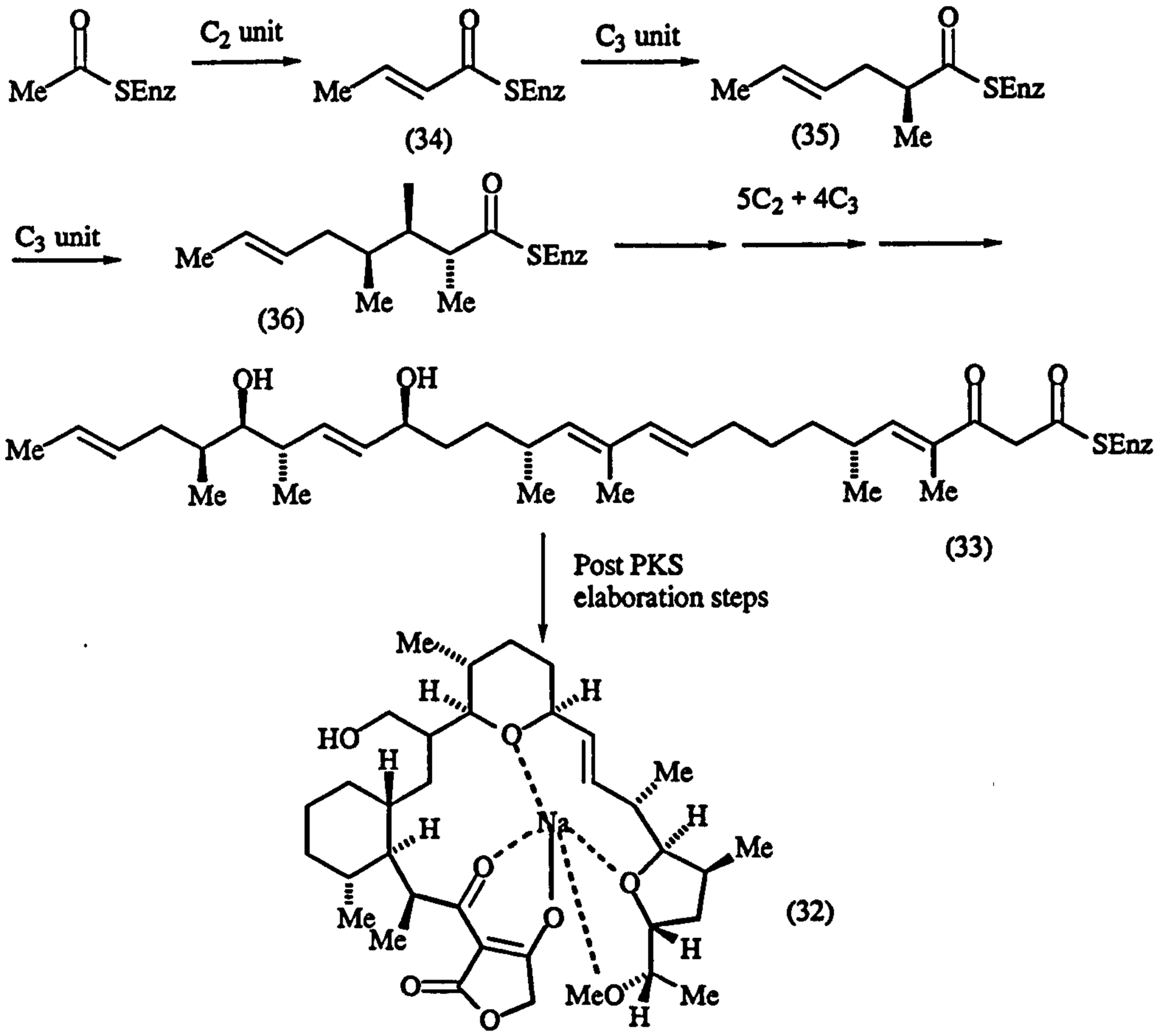
polyketides. In 1988, he reported the intact incorporation of the N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-(2*S*,3*R*)-3-hydroxy-2-methylpentanoic acid into nargenicin (30), in *Nocardia agentinesis* (Scheme 1.14),[22] and in 1995, the intact incorporation of the double ^{13}C-labelled pentaketide thioester (31) was described.[23]



Scheme 1.14: Incorporation of the diketide and pentaketide into nargenicin, produced by *N. agentinesis*.

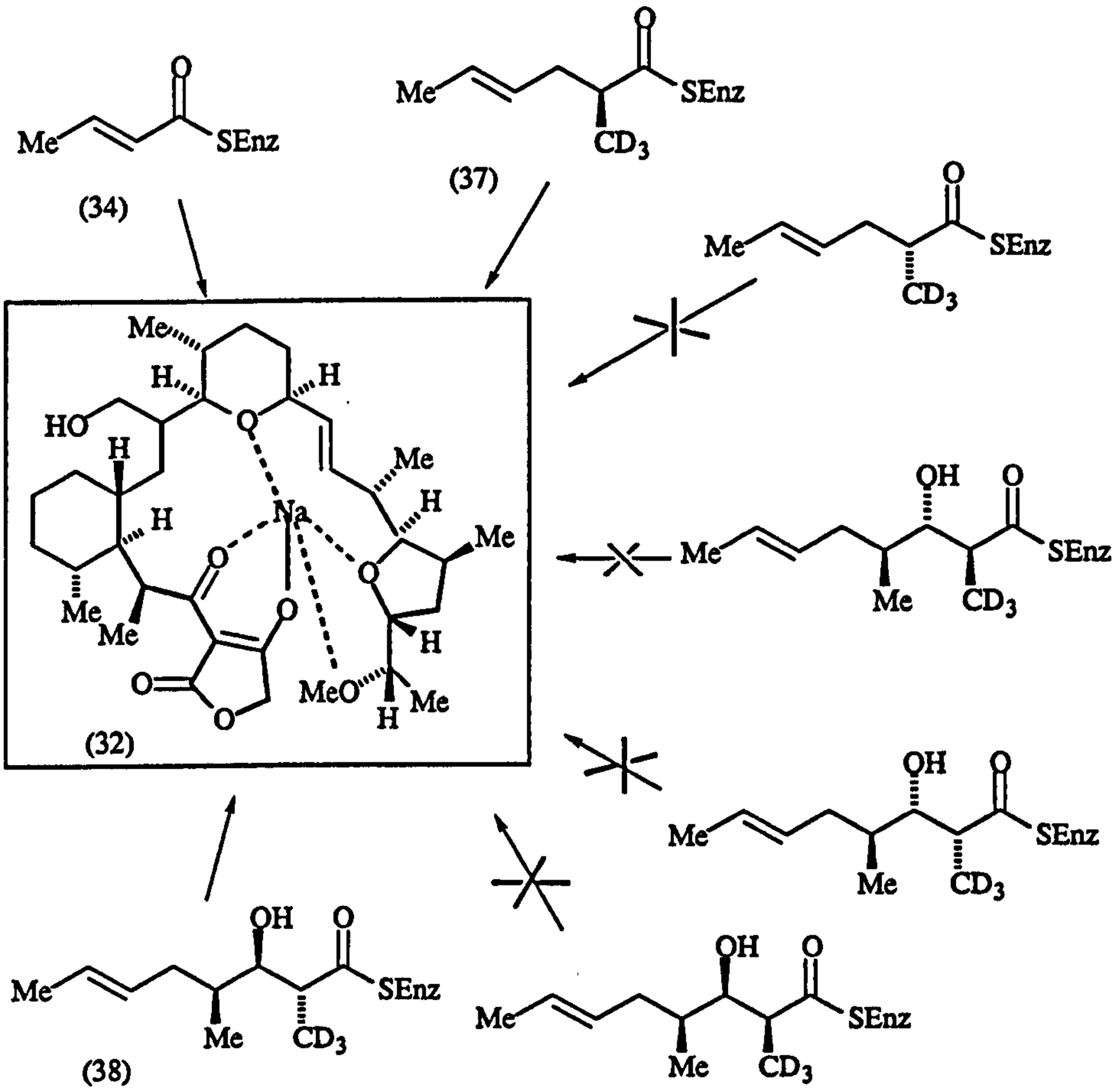
Tetronasin (32),[24] an antibiotic produced by *Streptomyces longisporoflavus*, is of commercial interest as a growth accelerator in cattle, and as an antiparasitic agent.[25,26] Preliminary biosynthetic studies, using acetate and propionate precursors labelled with ^{13}C, ^{2}H, and ^{18}O, have shown the carbon skeleton of tetronasin to be made up of seven acetate and six propionate units linked in a head to tail fashion, typical of a polyketide pathway. The O-methyl has been shown to be derived from S-adenosylmethionine, but the origin of the C_2 unit of C-33, C-34 remains unclear.[27,28]

A biosynthetic pathway was proposed, in which the linear polyketide intermediate (33) is assembled by a PKS, via a sequence of enzyme bound intermediates (Scheme 1.15). From acetyl CoA, twelve extension cycles with varying degrees of reduction are required to produce (33). It was suggested that if chain assembly was to follow a processive mode, in which, after each condensation, the required reductive modifications take place before the next condensation, the first three intermediates on the biosynthetic pathway would be (34), (35), and (36).



Scheme 1.15: A proposed biosynthetic pathway for tetronasin.

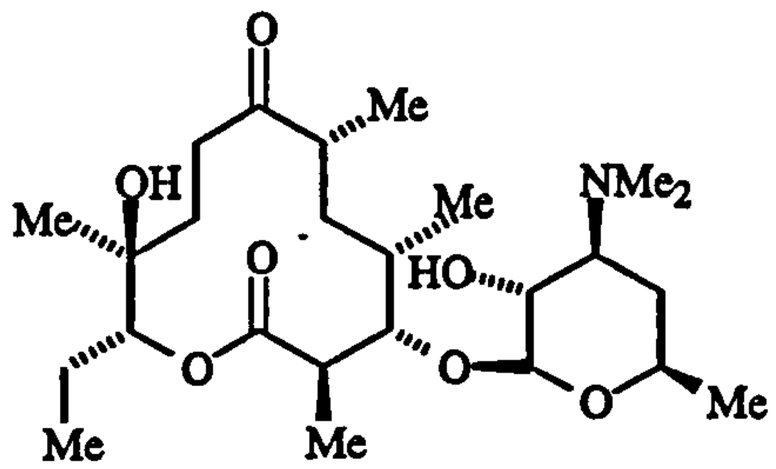
In order to investigate this hypothesis, Staunton and coworkers prepared a range of deuterated isotopomers of the putative diketide (34),[29,30] triketide (35),[29-31] and tetraketide (36).[31-33] They reported the intact incorporation of the deuterated triketide (37) into C-27 of tetronasin on administration to *Streptomyces longisporoflavus*, thus supporting the hypothesis that the proposed triketide (35) was a biosynthetic intermediate, and therefore by implication, so was the diketide (34) (Scheme 1.16).[29] They later noted that the isotopically label from the deuterated tetraketide (38) was incorporated intact into C-27 of tetronasin. It was also shown that only the correct diastereoisomer (36) was incorporated intact, whereas the other three diastereoisomers were degraded.[33]



Scheme 1.16: Incorporation of chain elongation intermediates into tetronasin.

A range of structural analogues of the diketide, triketide, and tetraketide were also synthesised, including fluorinated analogues, or methyl groups replaced by ethyl, *iso*-propyl, or benzyl moieties.[30,32] Staunton and coworkers reported that the precursor analogues containing smaller groups, *e.g.* fluorine or ethyl, were incorporated intact giving the corresponding analogues of tetronasin. However, the analogues containing larger, unnatural groups, *e.g.* iso-propyl or benzyl, did not produce any structural analogues of tetronasin.[34]

In summary, the findings that diketide, triketide, tetraketide and pentaketide chain elongation intermediates, administered as their N-acetylcysteamine thioesters, can be incorporated intact has provided a powerful tool for probing the detailed sequence of the biosynthesis of many polyketide natural products. Other examples where this approach has been successfully used include the elucidation of the biosynthesis of the bacterial metabolite methymycin (39),[35] and the fungal metabolites aspyrone (40)[36-40] and dehydrocurvularin (41).[41,42]



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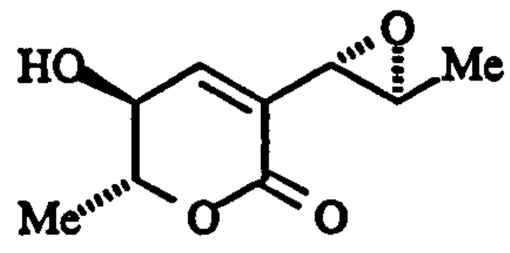

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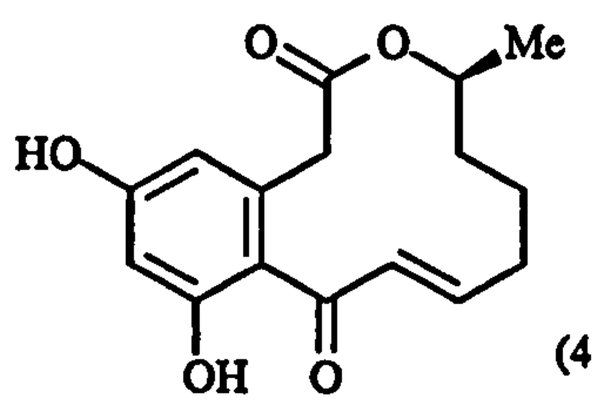
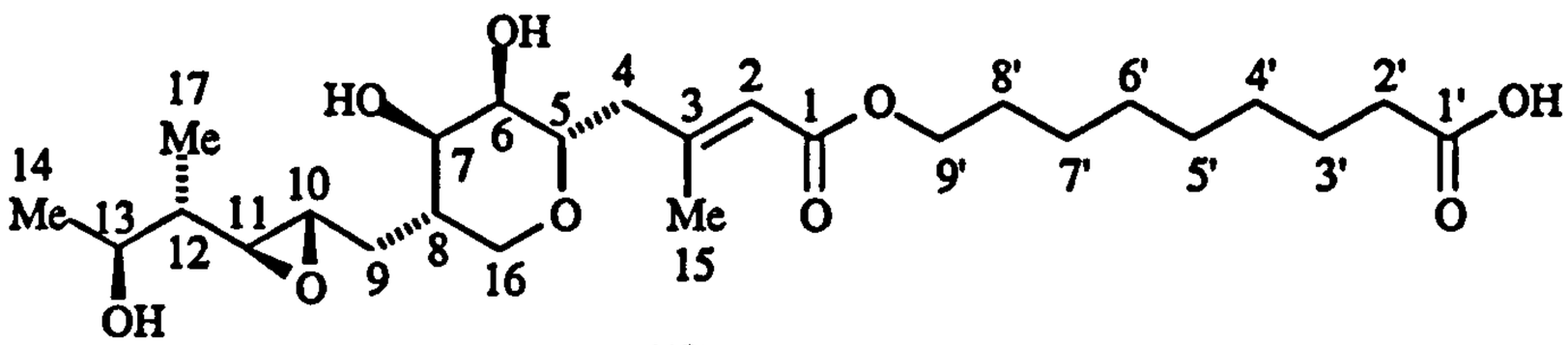


(41)

1.4 Pseudomonic acid: Discovery

The antagonistic effects of *Pseudomonas fluorescens*, a Gram negative bacterium[43] upon various fungi, bacteria and yeasts were first noted in 1887 by Garre.[44] Later, Chain and coworkers published the antibiotic activity of cultures and extracts of *Pseudomonas fluorescens*.[45] In 1971, a metabolite was isolated from submerged fermentations of *Pseudomonas fluorescens* NCIB 10586, which was responsible for nearly all of the biological activity.[46] This metabolite was named pseudomonic acid A (42).

Structural elucidation studies by Chain and Mellows[47,48] revealed that pseudomonic acid (42) has an unusual carbon framework, based upon a vicinally dihydroxylated pyran ring The side chain attached to C-8 contains an epoxide at C-10,11, and an alcohol at C-13. The side chain attached to C-5 contains an α,β unsaturated acid, which is condensed with 9-hydroxynonanoic acid, to give the ester. Further X-ray crystallography and nmr studies by Clayton and coworkers showed the absolute stereochemistry of pseudomonic acid A (42) to be as shown below.[49]



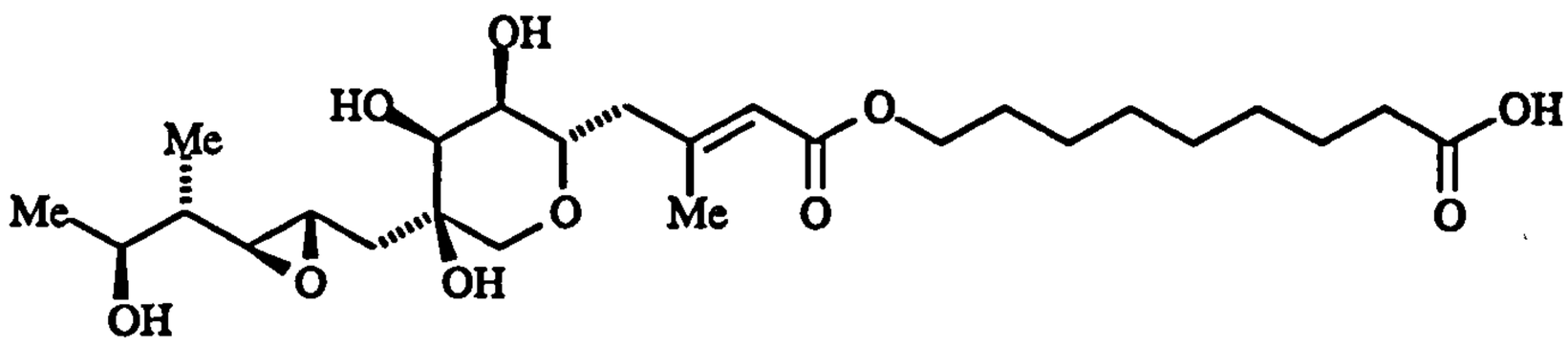
(42)

1.5 Pseudomonic acid: Other metabolites

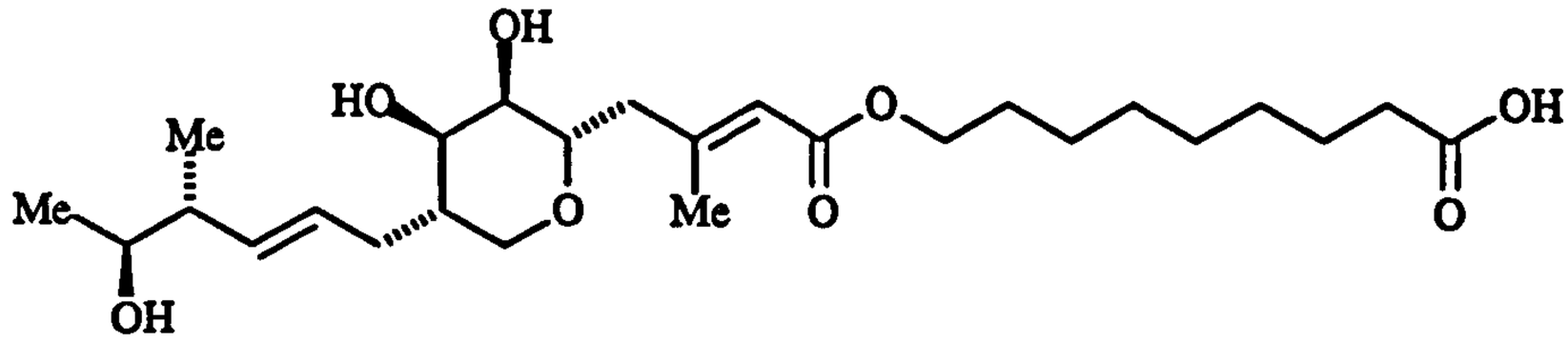
Following extensive purification of the crude antibiotic mixture, isolated from *Pseudomonas fluorescens* by Chain and coworkers in 1971,[46] essentially all of the

antibacterial activity was found to be associated with a family of structurally related substances, the 'pseudomonic acids'.

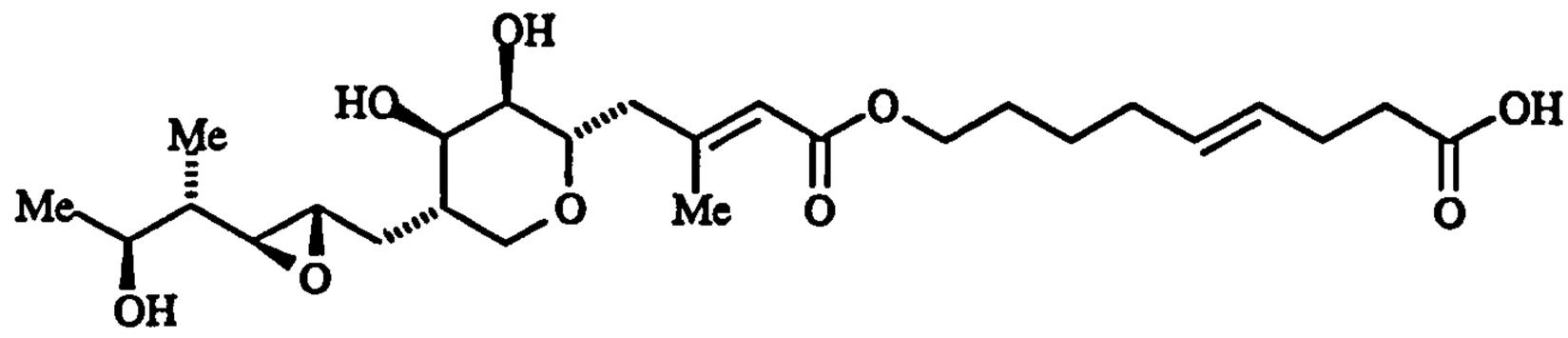
Pseudomonic acid A, which exhibits the strongest antibiotic activity, represents 95% of the crude mixture, and was the first member of the family to be isolated,[46] and structurally characterised.[49] Further work with *Pseudomonas fluorescens* NCIB 10586 has led to the discovery and isolation of four other members of the pseudomonic acid family: Pseudomonic acids B (43),[50] C (44),[51] D (45),[52] and E (46).[53] Structural elucidation studies have shown that pseudomonic acid B differs from pseudomonic acid A in that it contains a hydroxyl at C-8, whereas pseudomonic acid C differs in that it has a double bond in place of the epoxide functionality at C-10,11. Pseudomonic acids D and E differ from pseudomonic acid A in the nature of the side chain ester. In pseudomonic acid A there is a 9-hydroxynonanoic acid side chain, in pseudomonic acid D there is a 9-hydroxynon-4-enoic acid side chain, and in pseudomonic acid E there is a 10-hydroxydecanoic acid side chain.



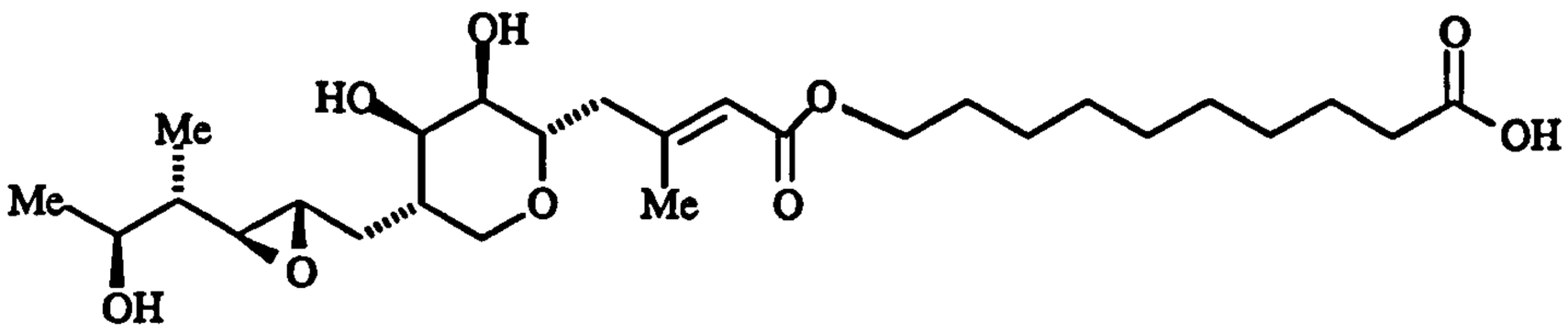
Pseudomonic acid B (43)



Pseudomonic acid C (44)



Pseudomonic acid D (45)

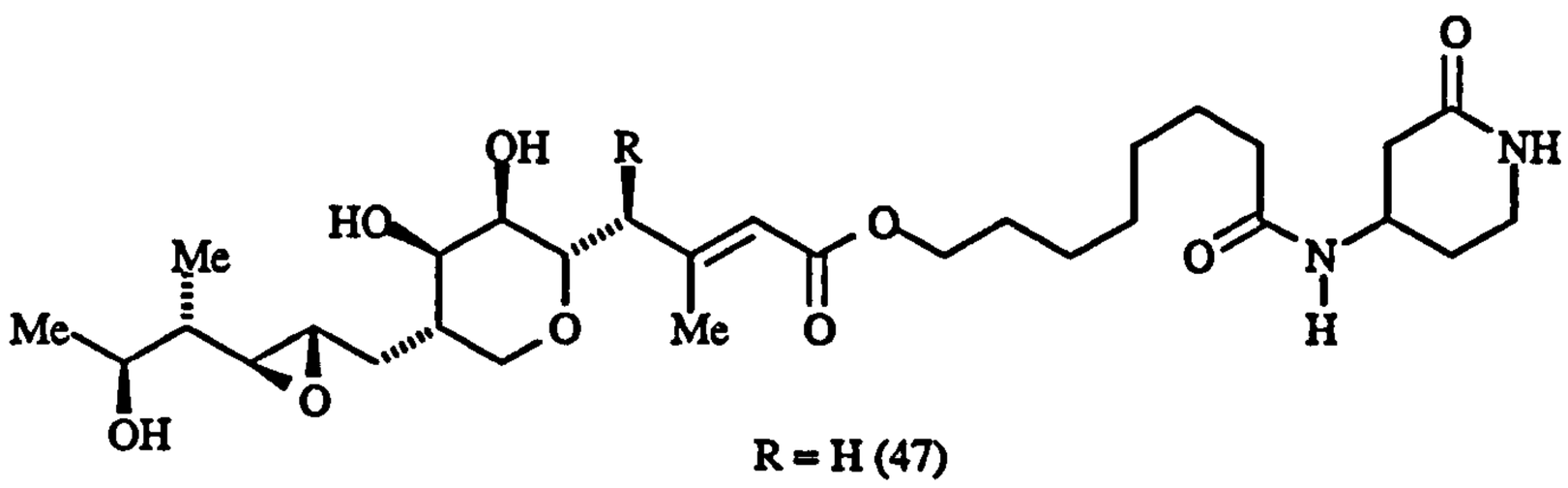


Pseudomonic acid E (46)

Fig. 1.5: Other members of the pseudomonic acid family.

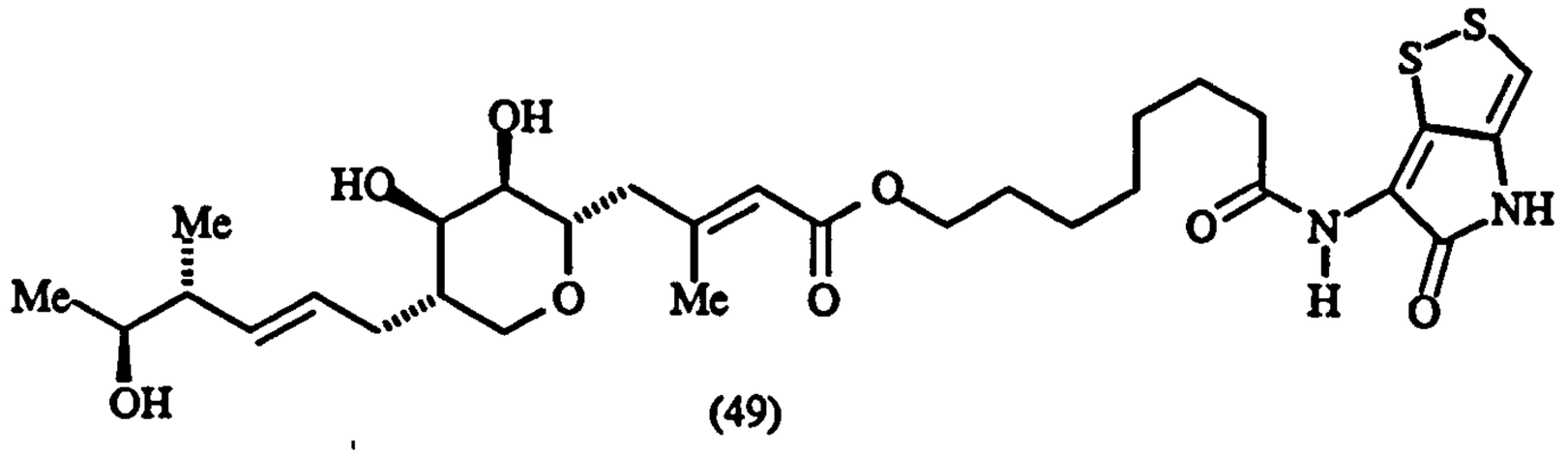
These minor metabolites have biological activities similar to or less than pseudomonic acid A. Throughout the remainder of this thesis pseudomonic acid will refer to the major metabolite pseudomonic acid A.

Many other structurally related derivatives of pseudomonic acid have been isolated in recent years. Two novel pseudomonic acid derivatives (47) and (48) were isolated from an *Alteromonas species* bacterium, which is isolated from the marine sponge *Darwinella rosacea*.[54] In both metabolites, the 9-hydroxynonanoic acid side chain has been replaced by an 8-hydroxyoctanamide side chain. The derivative (48) also possesses a 4β-hydroxyl group.



R = H (47)
R = OH (48)

Thiomarinol (49) has been isolated from a marine bacterium *Alteromonas reva sp. nov.* SANK 73390, and found to have stronger inhibitory antimicrobial activity against Gram positive and Gram negative bacteria, as compared with pseudomonic acid.[55] Structural elucidation of thiomarinol (49) revealed that it was a hybrid of two types of antibiotics: pseudomonic acid and pyrrothines.



(49)

This raises some interesting evolutionary questions concerning the biosynthesis of such hybrid metabolites, as well as answering the questions posed concerning its antimicrobial activity.

1.6 Pseudomonic acid: Biological activity and mode of action

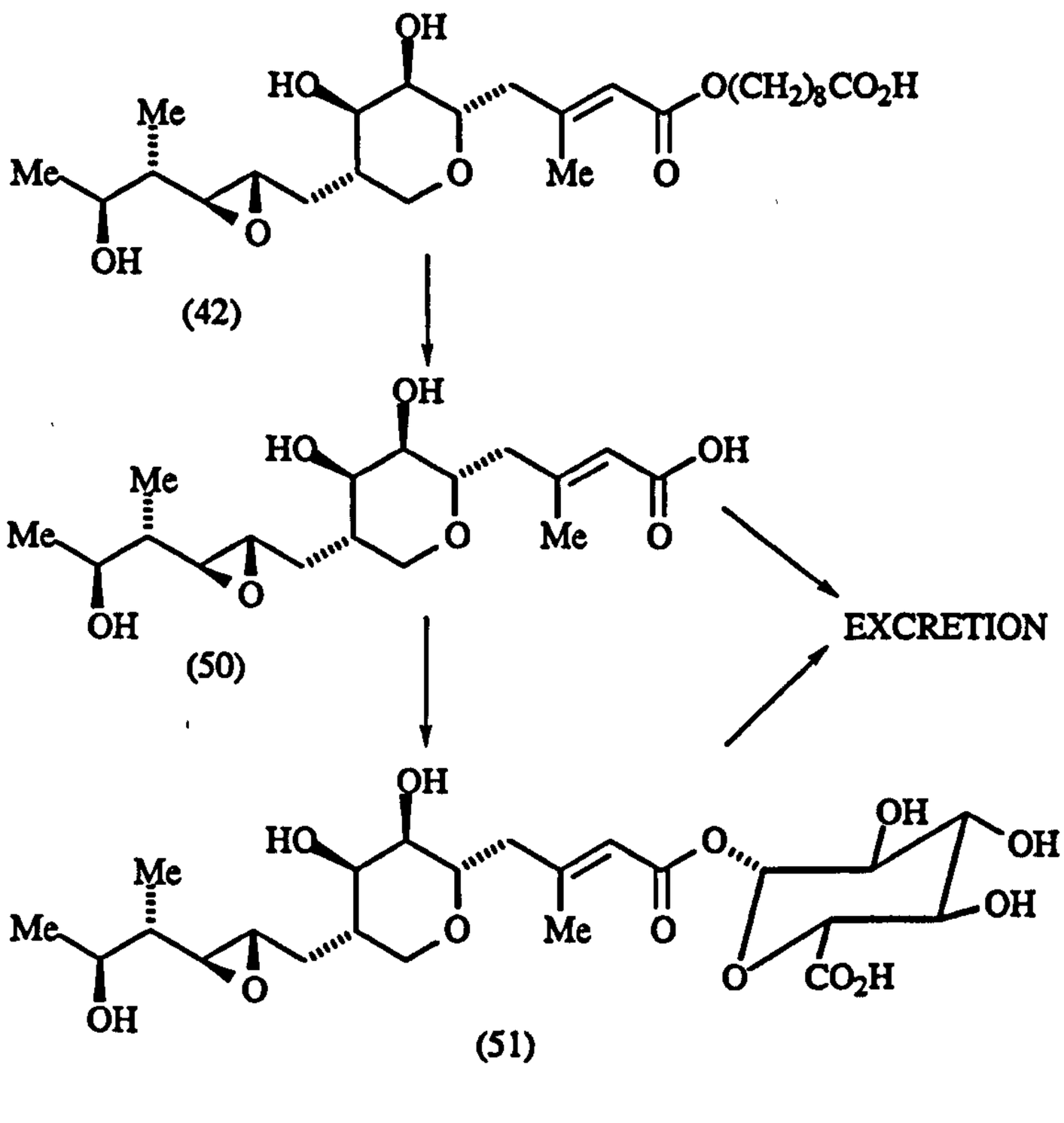
Currently, pseudomonic acid is marketed under the generic name, mupirocin, as the active component (2% pseudomonic acid in a polyethylene glycol base) in the topical skin cream Bactroban. *In vitro* tests have shown that its activity is enhanced in an acid medium, being 2 to 4 times more active at pH 6 than at pH 7.4, and being even less active

at pH 8. The greater activity at acid pH reinforces the suitability of Bactroban for the use in the acid environment of the skin.[56]

The biological activity of Bactroban was found to be restricted to the pH range 4 to 9,[46] but within these limits it has a wide spectrum of activity. It shows high levels of activity against not only the Gram-positive cocci most commonly associated with skin infections, *i.e.* staphylococci and streptococci species,[56] but also a variety of other potential pathogens, both Gram-positive and Gram-negative, aerobic and anaerobic.[56]

Another advantage of Bactroban is that, whilst being highly active against staphylococci and streptococci responsible for the majority of skin pathogens, it shows a relatively low level of activity against those members of the normal skin flora, the skin commensals, whose presence is considered to make a positive contribution to the prevention of infection to the individual, such as corynebacteria, micrococci and anaerobic propionibacteria.[56]

Following oral or intravenous administration, pseudomonic acid is rapidly converted to the inactive monic acid (50), which is eliminated primarily in the urine.[57] It has been shown that subcutaneous administration to mammals resulted in rapid excretion, via hydrolysis, of mainly monic acid β-glucoronide (51) (Scheme 1.17).[58]



Scheme 1.17: Metabolism and excretion of pseudomonic acid.

These observations indicate that any pseudomonic acid or monic acid, which reaches the systemic circulation should be quickly metabolised and excreted from the body. Whilst it is desirable that a topical antibiotic should not be absorbed systemically, it is also important that it should penetrate the superficial layers of the skin itself.

In summary, the pharmacological characteristics of pseudomonic acid A that make it particularly suitable for topical use include

(i) it is stable and persistent on the skin, and does not disturb the skin commensals,

(ii) it achieves good penetration of the superficial layers of the skin, but

(iii) shows negligible systemic absorption,

(iv) rapid systemic metabolism makes it unsuitable for systemic use.

The mode of action of pseudomonic acid has been studied in *Staphylococcus aureus* and *Escherichia coli*, in which the primary effect was found to be inhibition of protein synthesis. Bactroban, therefore, has an unusual mode of action. It exerts its antibacterial activity by blocking protein synthesis within the bacterial cell.[59]

Subsequent studies identified the enzyme isoleucyl t-RNA synthetase as a target of the antibiotic in the bacterial cell.[60] This enzyme catalyses the formation of isoleucyl t-RNA, from t-RNA and isoleucine (52), which serves to transport isoleucine to the ribosome for the insertion into the growing protein chain. The action of Bactroban was shown later by Hughes and Mellows[60] to be the result of pseudomonic acid acting as a competitive inhibitor of the enzyme isoleucyl t-RNA synthetase, by competing with isoleucine for the enzyme binding sites. This will therefore halt protein synthesis within the bacterial cell.

It has been suggested that the active part of pseudomonic acid is the terminus of the C-8 side chain, which has a similar carbon skeleton as isoleucine, and so may compete with isoleucine for the single isoleucine binding site on the enzyme,[60] but has a more potent affinity for the enzyme than does isoleucine by 6 orders of magnitude.

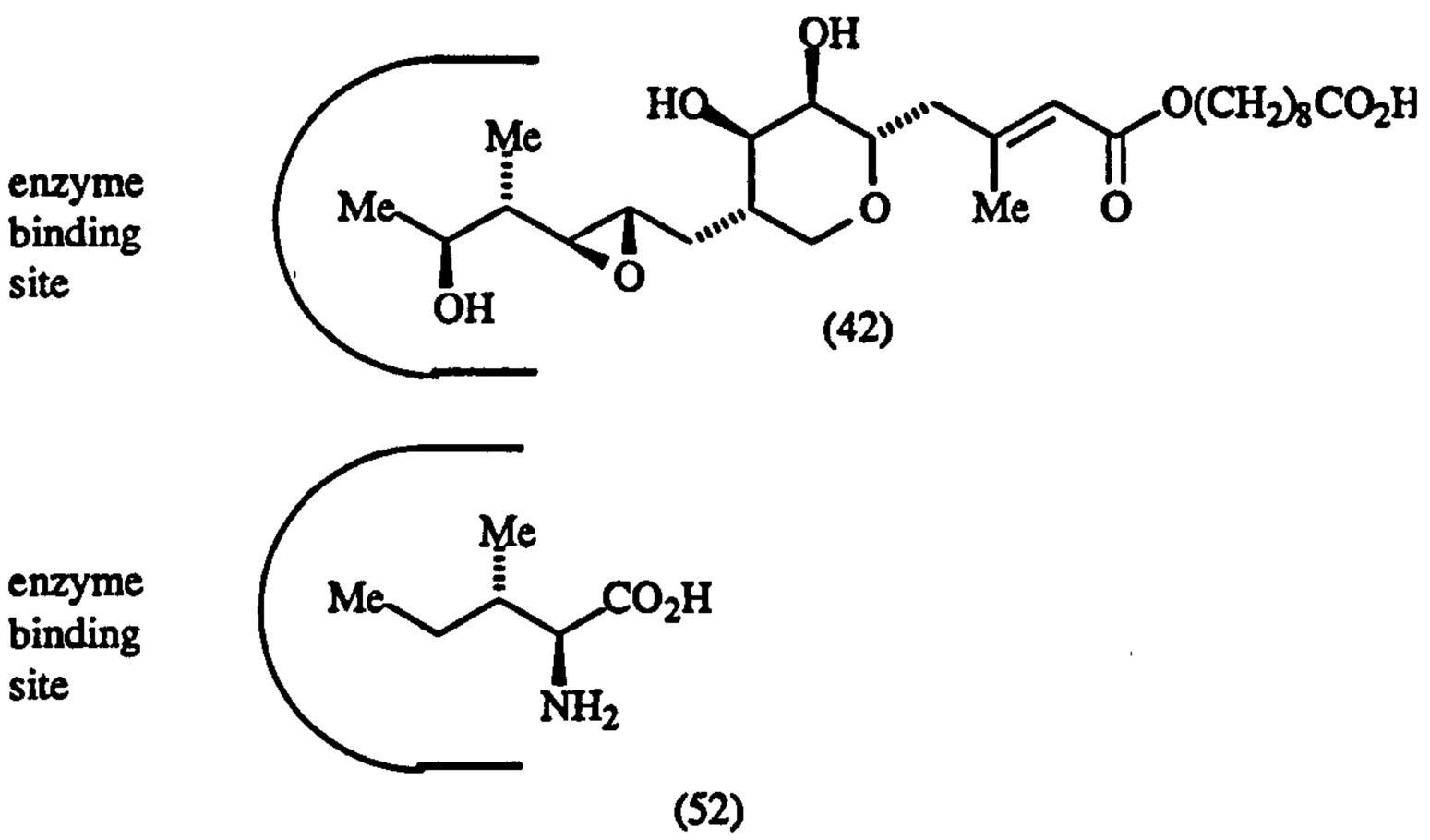
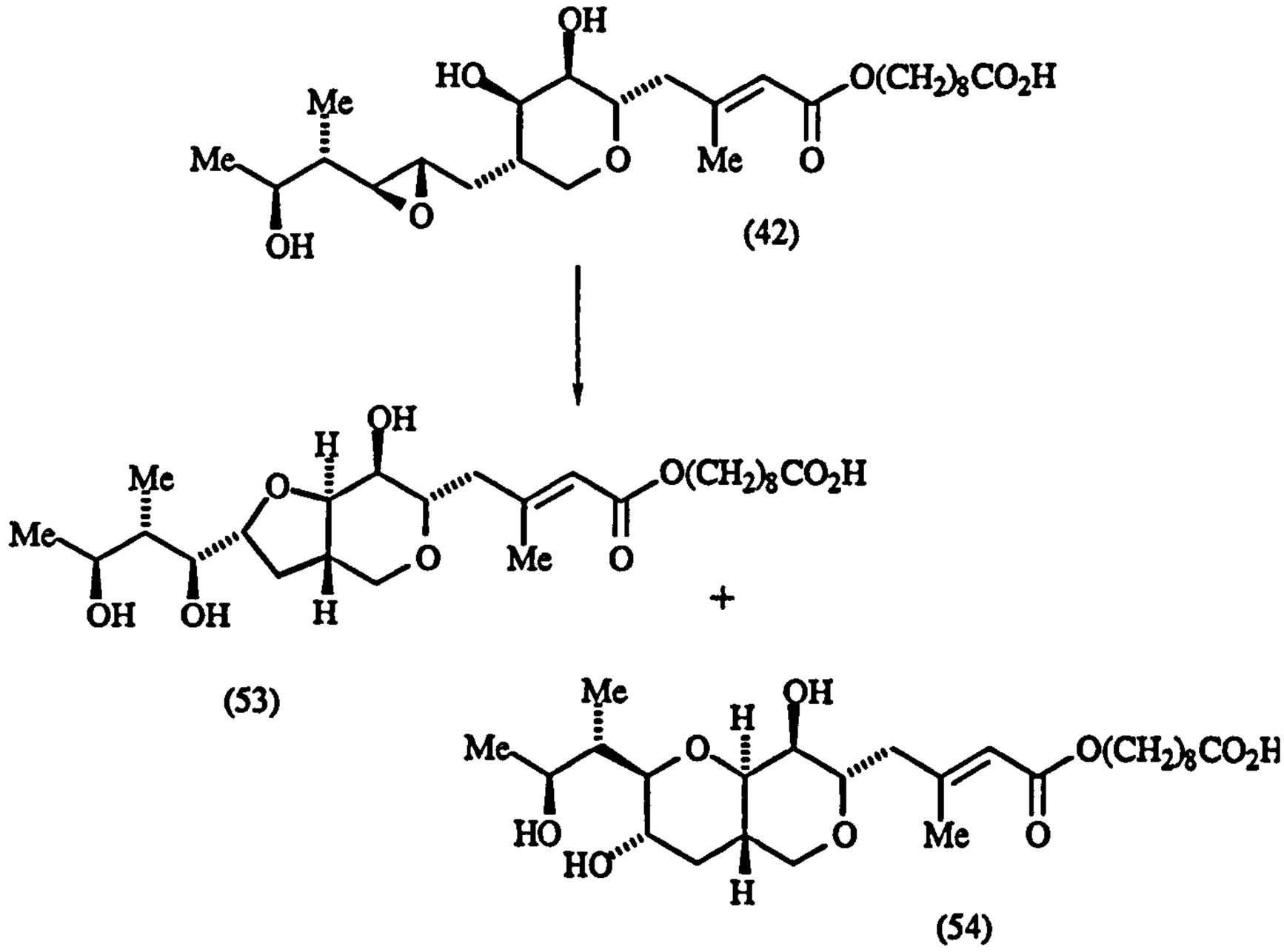


Fig. 1.6: Structural similarity of pseudomonic acid and isoleucine.

As a result, at low concentration, pseudomonic acid slows down bacterial protein synthesis, and produces bacteriostasis.[58] However, at high concentrations, as are achieved with Bactroban therapy, it blocks protein synthesis with bacteriocidal results.[61]

As already mentioned, the biological activity is only observed between the pH limits of 4 and 9. This results from the instability of pseudomonic acid to strong base and acid.[62] At low pH, rapid rearrangement occurs, leading to a 1.6:1 ratio of the isomeric compounds (53) and (54) (Scheme 1.18). Under strongly alkaline conditions, a slower rearrangement occurs, leading to a 3.7:1 ratio (53) and (54).

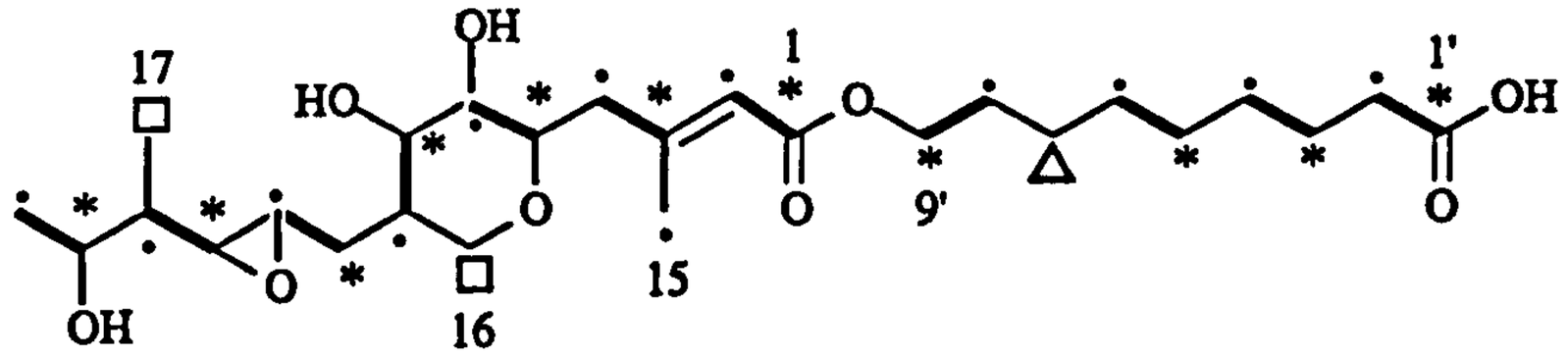


Scheme 1.18: Acid and base rearrangement of pseudomonic acid.

The fact that it is the C-8 side chain that is disrupted on addition of acid or base, supports the hypothesis that it is this part of pseudomonic acid that is responsible for the observed biological activity. However, Klein and coworkers,[63] and Yanagisawa and coworkers[64] noted that the remaining structure of pseudomonic acid differs greatly from isoleucine, but it is indispensable for the inhibition of isoleucyl t-RNA synthetase by pseudomonic acid. This suggested that, in addition to the putative isoleucine binding site on isoleucyl t-RNA synthetase, an additional site must participate in binding the remainder of the pseudomonic acid. It was concluded that pseudomonic acid is an inhibitor of isoleucyl t-RNA synthetase, possessing characteristic features of both an isoleucine analogue, and an ATP analogue, *e.g.* isoleucyl adenylate.[64]

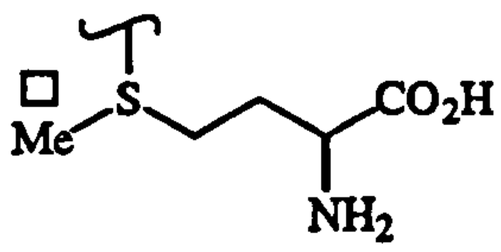
1.7 Pseudomonic acid: Biosynthetic studies

The nmr techniques described in section 1.2 were used in the initial investigations into the biosynthetic origins of the pseudomonic acids.[65] These studies involved feeding variously isotopically labelled acetates, propionates, and [methyl-^{13}C]-L-methionine to *Pseudomonas fluorescens*, and determination of the resultant labelling pattern in pseudomonic acid (Fig. 1.7).



Key: $CH_3\overset{*}{C}O_2Na$ [1-^{13}C]acetate CH_3—CO_2Na [1,2-$^{13}C_2$]acetate

$CH_3\overset{\bullet}{C}O_2Na$ [2-^{13}C]acetate $CH_3CH_2\overset{\triangle}{C}O_2Na$ [1-^{13}C]propionate

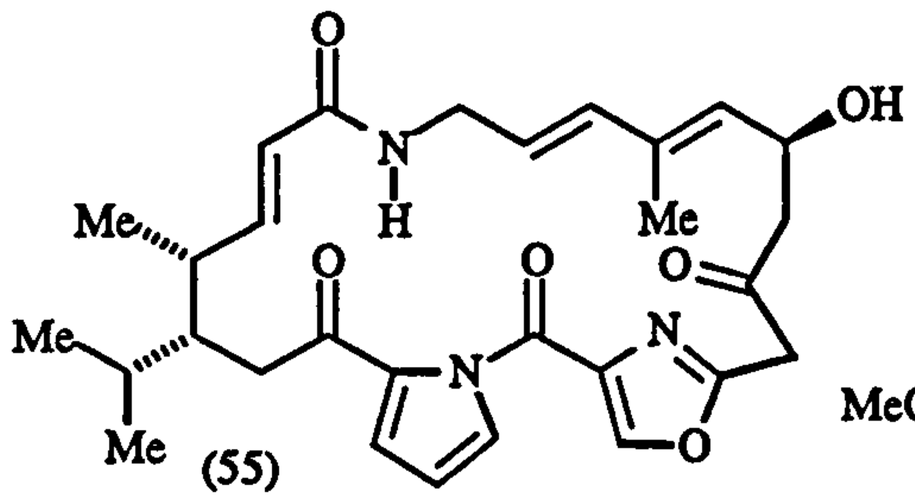


[methyl-^{13}C]-L-methionine

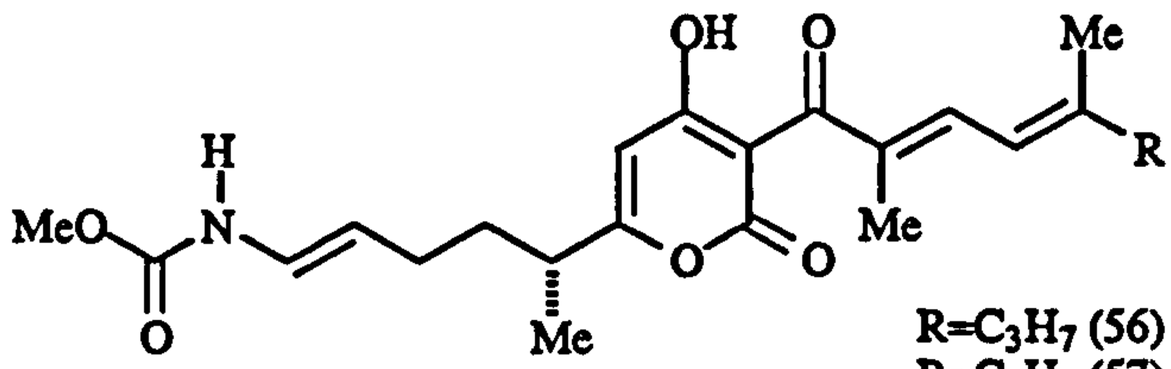
Fig. 1.7: Isotope labelling pattern in pseudomonic acid from feeding labelled precursors to *Pseudomonas fluorescens*.

The incorporation of acetate units in a 'head to tail' manner along much of the carbon framework appeared to be consistent with a polyketide/ fatty acid biogenesis. However, there were notable inconsistencies.

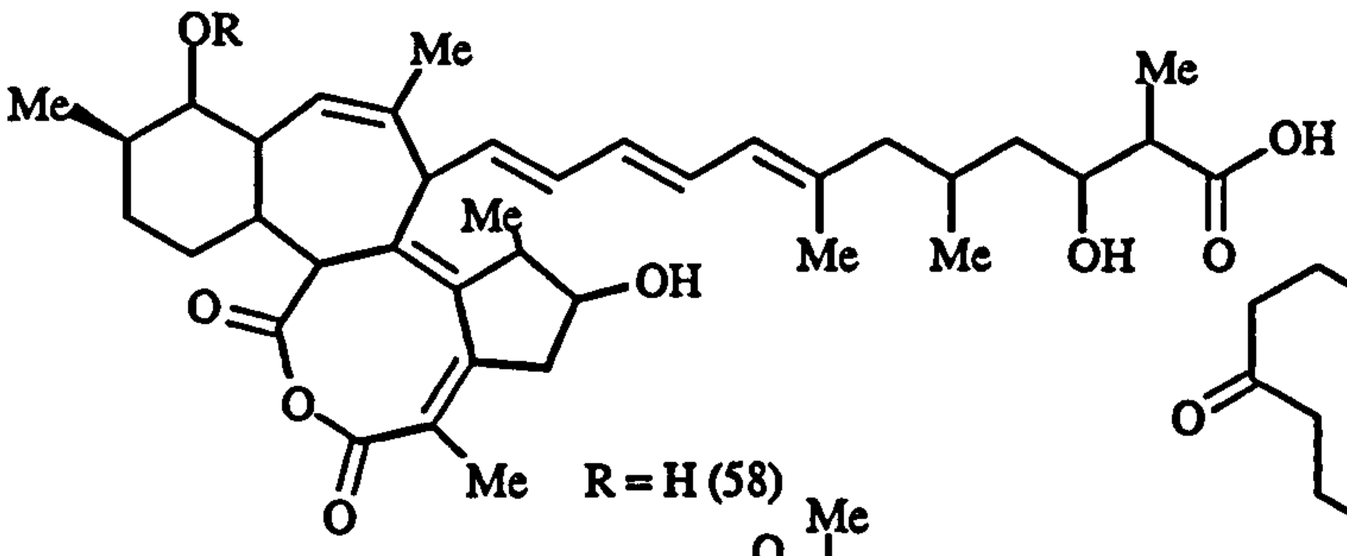
Firstly, whilst C-16 and C-17 were found to be labelled from methionine, thus indicating methylation, via S-adenosylmethionine, the branched C-15 methyl group was found to originate from the C-2 of a cleaved acetate unit. When this incorporation of a cleaved acetate unit was first discovered, it was unique to pseudomonic acid biosynthesis. However, similar results have been reported more recently for the biosynthesis of virginamycin M_1 (55),[66] myxopyronin A (56) and B (57),[67] aurantin A (58) and B (59),[68] and myxovirescin (60).[69]



(55)



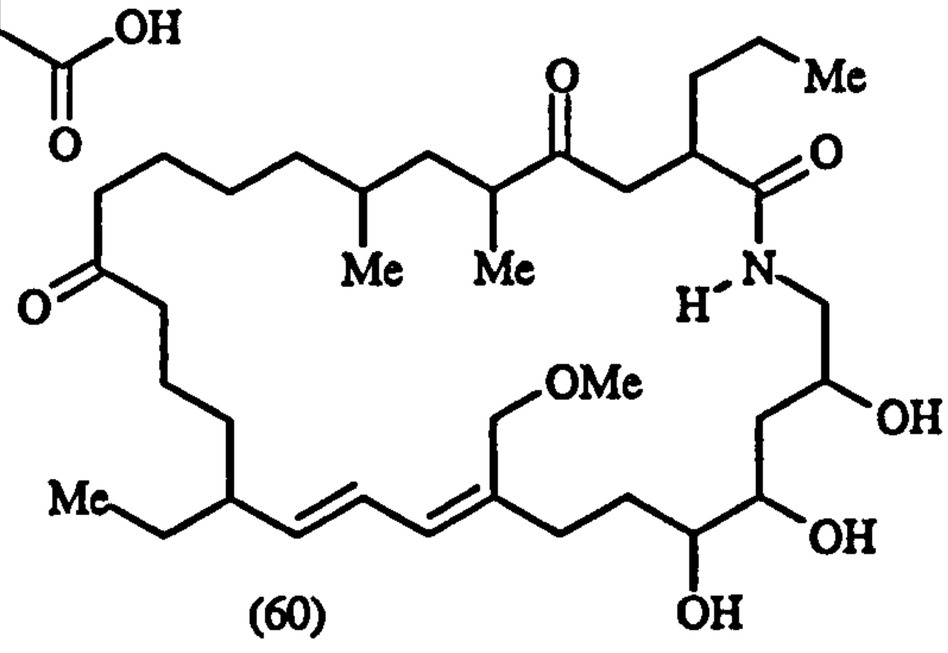
R=C_3H_7 (56)
R=C_4H_9 (57)



R = H (58)

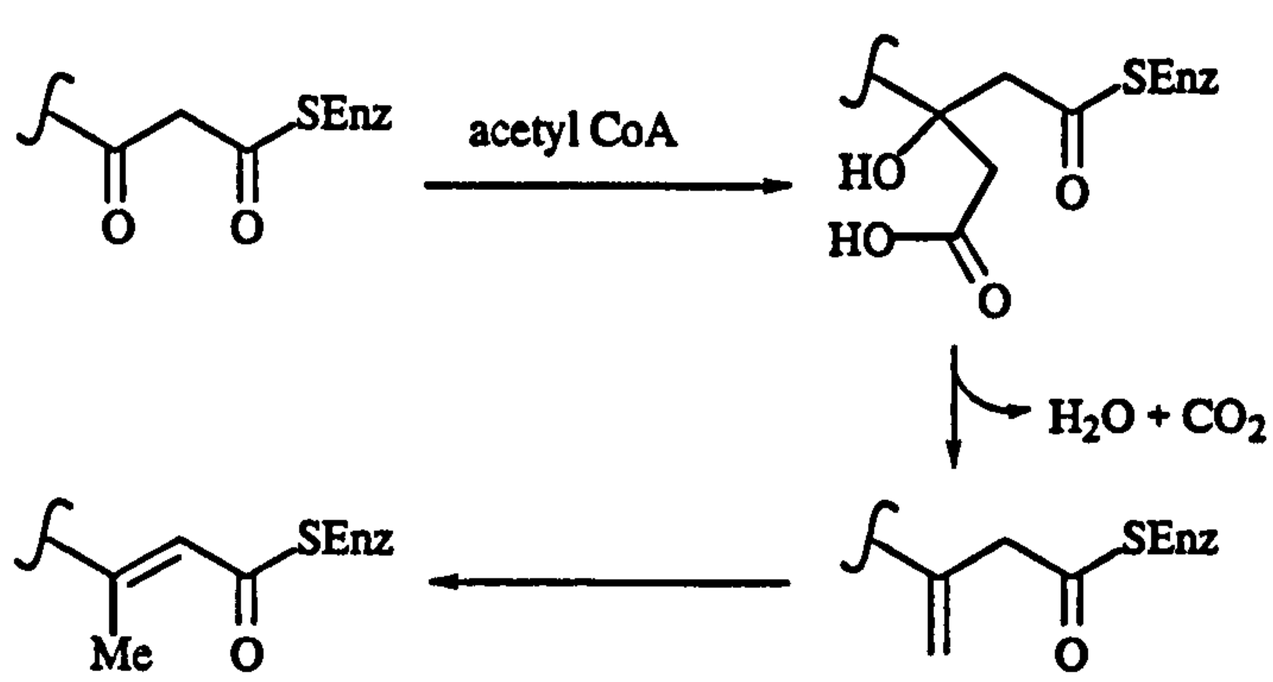
R =

(59)



(60)

This unusual labelling pattern may rationalised by invoking an aldol condensation of acetyl CoA onto a preformed polyketide chain, followed by thioester hydrolysis, decarboxylative dehydration and alkene isomerisation, as shown in Scheme 1.19.[66,70]

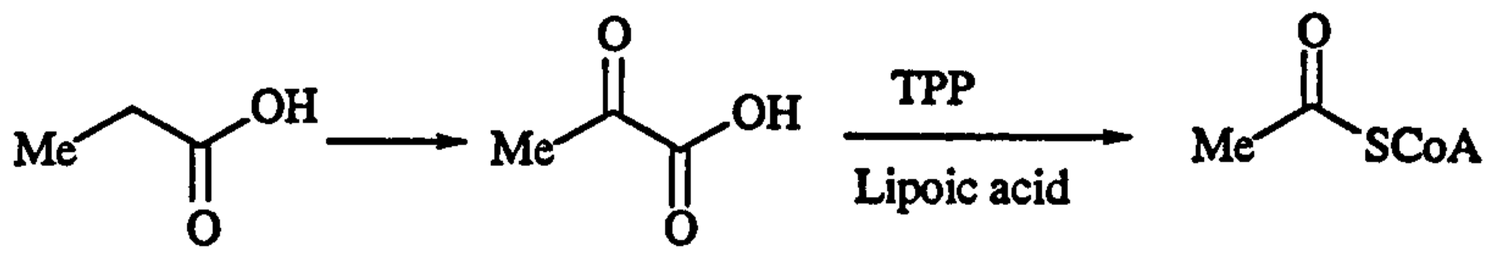


Scheme 1.19: Mechanism for the introduction of a cleaved acetate unit onto a polyketide chain.

The origin of the 9-hydroxynonanoic acid side chain was unusual. Intact acetate units were incorporated in the expected 'head to tail' manner at C-1'/C-2', C-3'/C-4', and C-5'/C-6'. However, C-8'/C-9', although also shown to be derived from acetate, it was apparent that the direction of acetate incorporation was opposite to the rest of the 9-hydroxynonanoic acid moiety, with C-7' acting as the pivotal point for this reversal. The carboxyl of an acetate unit was found to specifically label C-7', but the enrichment was only 50% that of the other carbons.

[1-^{13}C]Propionate was fed to *Pseudomonas fluorescens*, giving pseudomonic acid, which was found specifically labelled the C-7' position. However, on feeding [3-

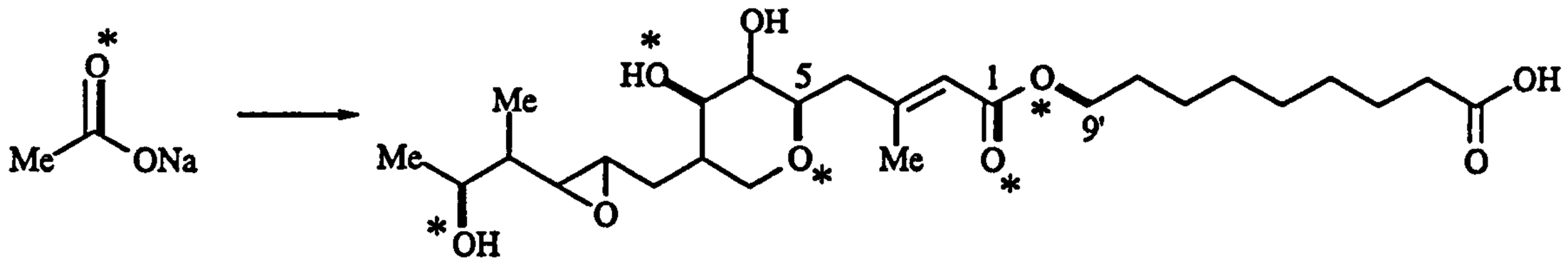
^{13}C]propionate, a labelling pattern identical to that of [2-^{13}C]acetate was obtained, indicating incorporation of propionate through to acetyl CoA, via α-oxidation to pyruvate (61) (Scheme 1.20).[65]



(61) (4)

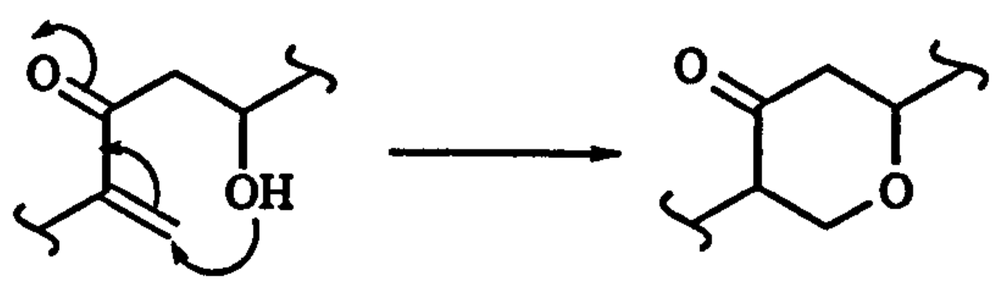
Scheme 1.20: α-oxidation of propionate to acetyl CoA, via pyruvate.

The fact that both C-1 and C-9' were derived from C-1 of acetate, implied that the ester functionality did not arise from a Baeyer-Villager type oxidation. To confirm that the bonding oxygen of the ester moiety was acetate in origin, [1-^{13}C,$^{18}O_2$]acetate was fed to *Pseudomonas fluorescens*.[71] Examination of the ^{13}C nmr spectrum of isolated pseudomonic acid confirmed that the (C-9')-O bond and the (C-1)-O bond are both derived from an intact acetate unit, as shown in Scheme 1.21.



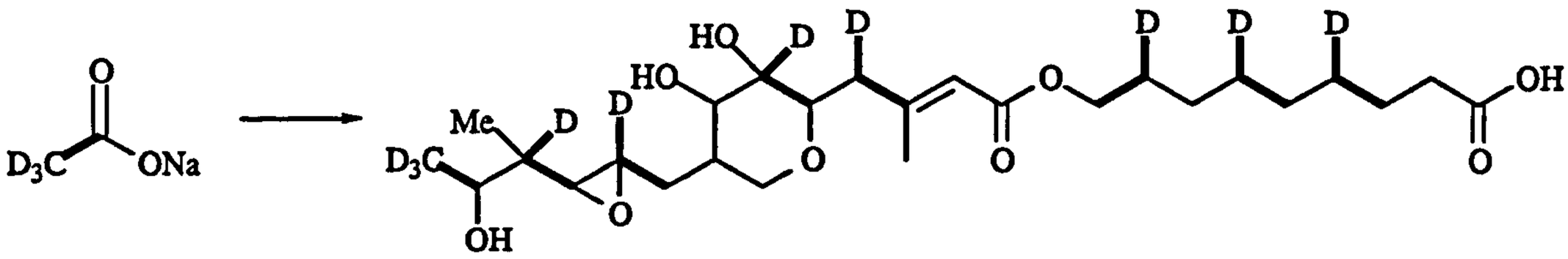
Scheme 1.21: Results of feeding [1-^{13}C,$^{18}O_2$]acetate to *Pseudomonas fluorescens* .

From this feeding study, it was shown that the oxygen at C-5 was derived from acetate. Simpson and Martin[71,72] proposed a mechanism for the formation of the tetrahydropyran ring, involving a conjugate addition type ring closure, as shown in Scheme 1.22.



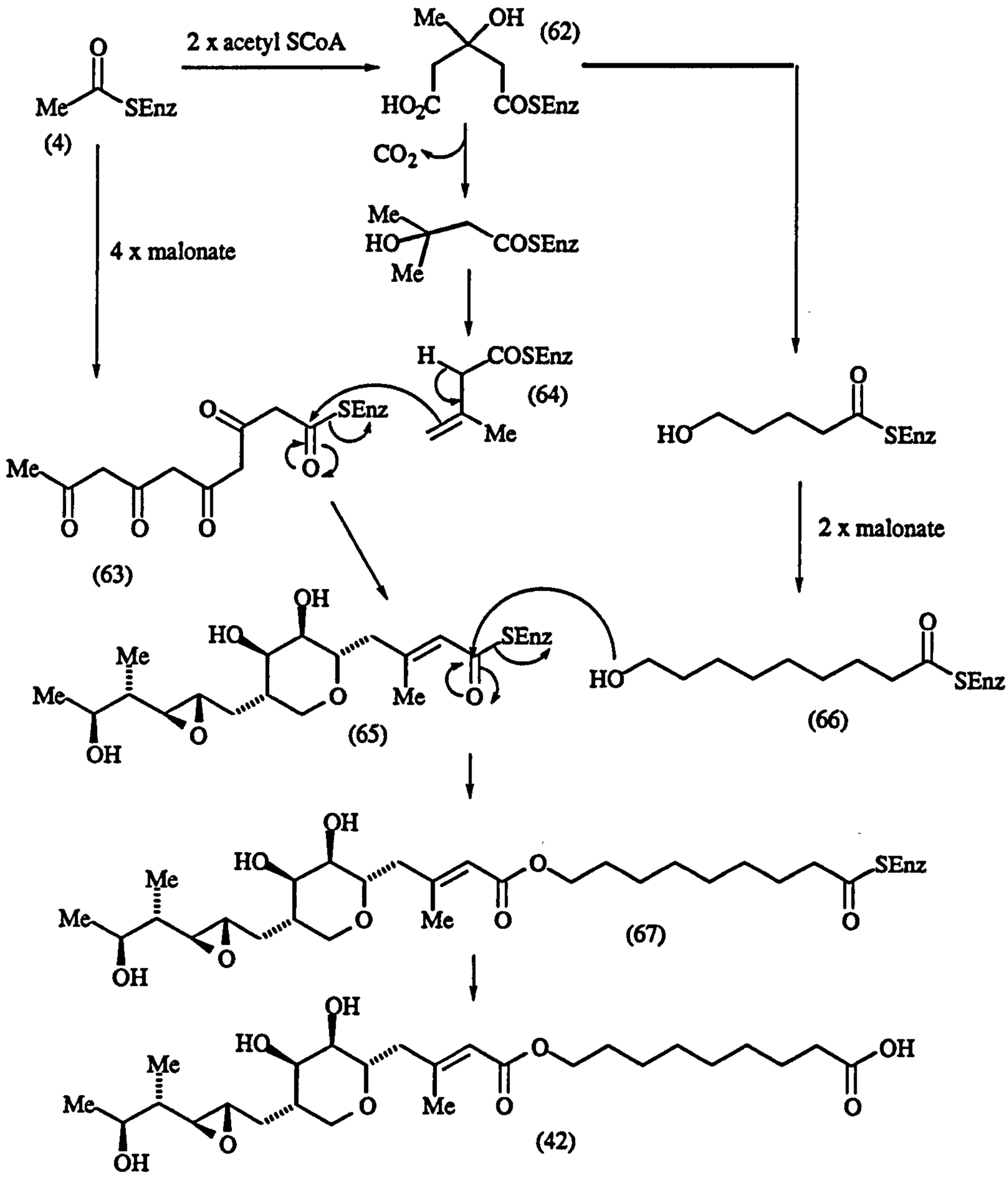
Scheme 1.22: Proposed mechanism for the tetrahydropyran ring formation.

This proposal is in accord with the results from feeding studies with [1-^{13}C,$^{2}H_3$] acetate to *Pseudomonas fluorescens* (Scheme 1.23).[71,72] The lack of a β-shift on the signal assigned to C-7 in the ^{13}C nmr spectrum of pseudomonic acid indicated the deuterium from acetate was not retained at C-8.



Scheme 1.23: Results of feeding [1-^{13}C,$^{2}H_3$]acetate to *Pseudomonas fluorescens* .

Mellows and coworkers[65] accounted for these anomalies by proposing a cascade pathway, involving β-hydroxy-β-methylglutarate (62), as shown in Scheme 1.24.

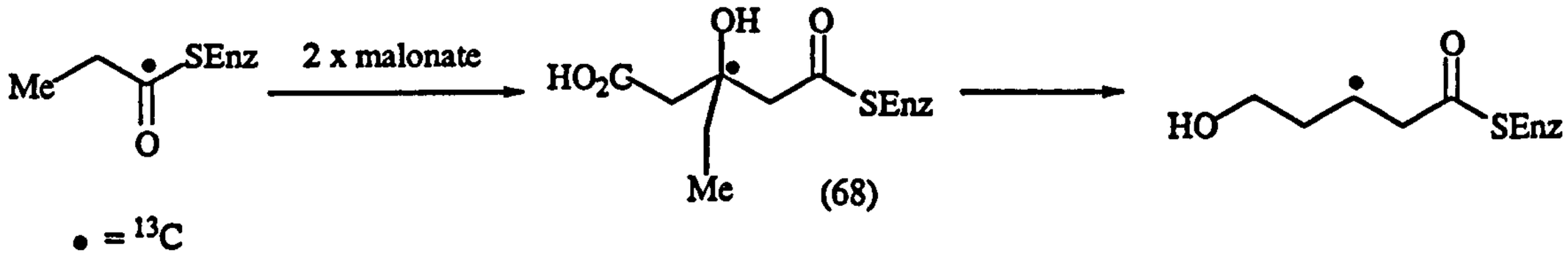


Scheme 1.24: Cascade hypothesis.

This theory also gave an explanation for C-15 being derived from a C-2 cleaved acetate unit. Hence, a polyketide derived C_{12} unit (63) then undergoes nucleophilic attack

from a C_5 unit (64), originating from β-hydroxy-β-methylglutarate. The resultant C_{17} thioester (65) was postulated to undergo nucleophilic attack from a C_9 moiety (66), also derived from β-hydroxy-β-methylglutarate followed by a fatty acid type chain elongation. Enzyme-bound pseudomonic acid (67) would be released to produce pseudomonic acid.

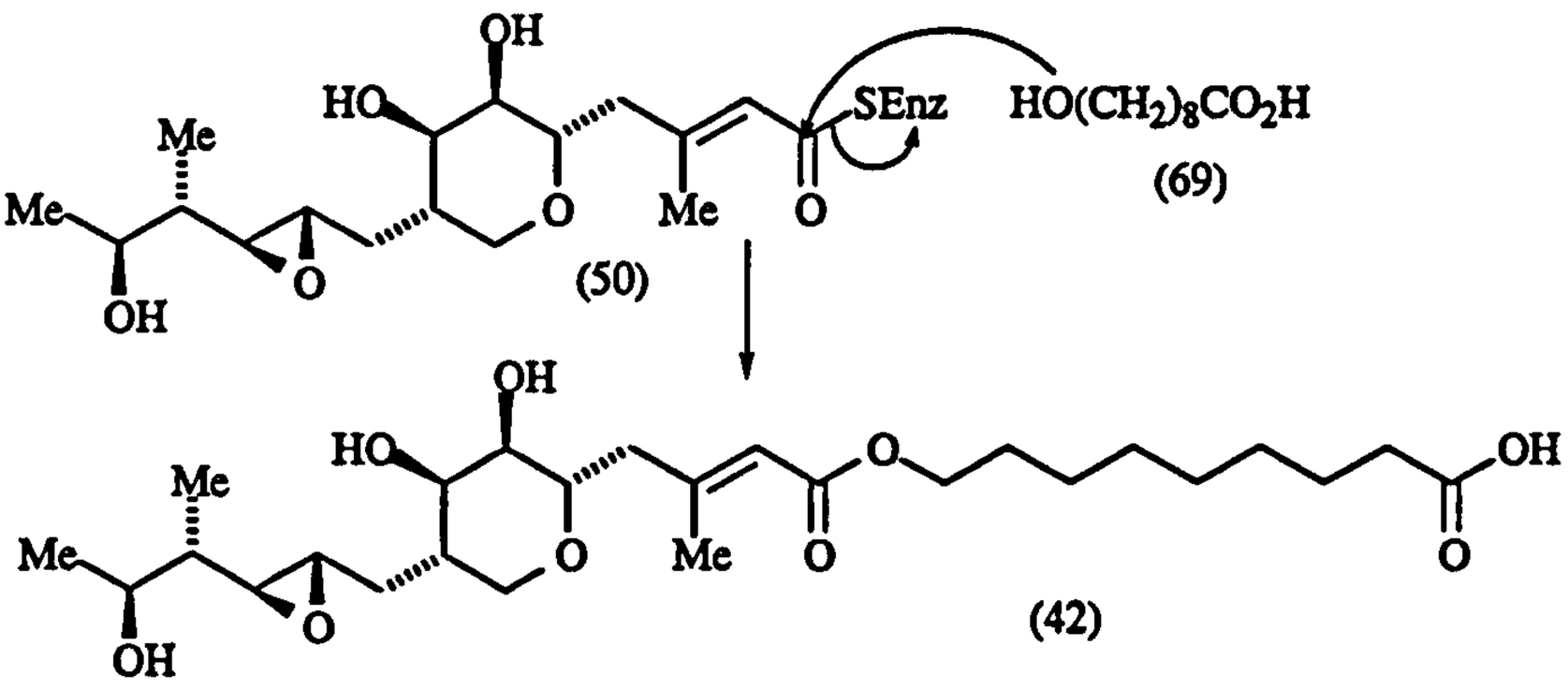
To explain the labelling pattern observed at C-7', competition between β-hydroxy-β-methylglutarate and β-hydroxy-β-ethylglutarate (68) was proposed, based on evidence of incorporation of homomevalonate into juvenile hormone JH II.[73] No experimental results were given to support this theory.



Scheme 1.25: β-Hydroxy-β-ethylglutarate as a precursor to the C_9 moiety.

This hypothesis was readdressed in 1988, when Mantle and Somner reported high incorporation of radiolabelled β-hydroxy-β-methylglutarate into pseudomonic acid.[74] This was interpreted as strong evidence for the cascade hypothesis, despite the lack of degradative studies to establish the position of the incorporated radio-label. Further work, by Simpson and Martin, using ^{13}C and $^{13}C_2$ labelled β-hydroxy-β-methylglutarate, indeed confirmed that carbon-13 was incorporated into pseudomonic acid. However, in both cases, it was shown that the labels incorporated throughout the molecule, with no evidence of specific incorporation.[71,72] This implied that β-hydroxy-β-methylglutarate had been degraded to acetate by β-oxidation, prior to incorporation into pseudomonic acid. Further work by Mantle and MacGeorge confirmed these findings.[75]

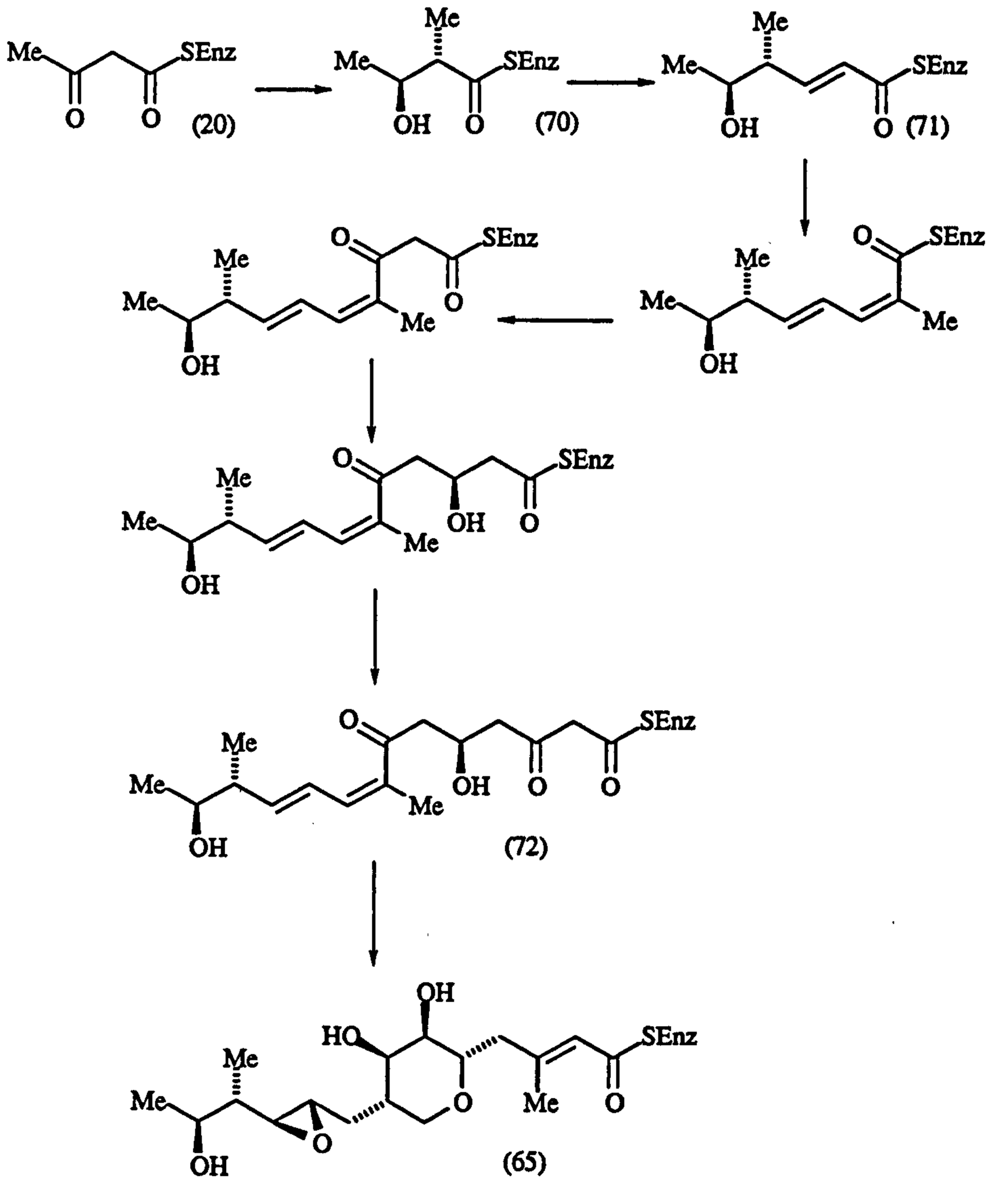
After these findings, a simpler hypothesis involving two distinct precursors, monic acid and 9-hydroxynonanoic acid (69), was postulated (Scheme 1.26).



Scheme 1.26: Postulated biosynthesis of pseudomonic acid via monic acid and 9-hydroxynonanoic acid.

Preliminary investigations into this hypothesis were carried out by Mantle and MacGeorge.[76] They obtained [16,17-$^{14}C_2$]monic acid, via feeding radiolabelled methionine to bacterial cultures, and subsequent saponification of the resultant radiolabelled methyl pseudomonate. However, on feeding [16,17-$^{14}C_2$]monic acid to cultures of *Pseudomonas fluorescens*, it was found that all of the radioactivity remained in the medium and did not cross cell walls. Therefore this hypothesis still needs to be proven.

From the results of feeding studies isotopically labelled precursors to *Pseudomonas fluorescens*, it has been established that the monic acid moiety of pseudomonic acid is derived from a series of acetate units in a 'head to tail' fashion. It has been suggested that monic acid is therefore polyketide in origin, and a proposed biosynthetic pathway is outlined in Scheme 1.27.



Scheme 1.27: Postulated processive mode of assembly of monic acid.

The initial acetate and malonate condensation produces enzyme bound acetoacetate (20), which undergoes methylation. Reduction of the β-keto function gives the (2*S*,3*S*)-3-hydroxy-2-methylbutanoate enzyme bound moiety (70), which on further condensation with malonate produces a hexanoyl moiety. A further reduction, and subsequent dehydration leads to the (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enoate enzyme bound intermediate (71). Further malonate condensations, together with the required reductive modifications, all being controlled by PKS enzymes, lead eventually to the putative heptaketide (72), prior to post-PKS translation to enzyme bound monic acid (65).

1.8 Aims

The overall aim of this project was to further investigate the biosynthetic pathway to pseudomonic acid, with particular emphasis on the assembly of the unusual 9-hydroxynonanoic acid fragment and its coupling with monic acid.

The approach required:

1) that an efficient, consistent production of pseudomonic acid by *Pseudomonas fluorescens* NCIB 10586 was established. Martin[71] and Sugden[77] had both previously reported problems with inconsistent production of pseudomonic acid. The optimum time for feeding isotopically labelled substrates to *Pseudomonas fluorescens* for maximum incorporation into pseudomonic acid needed to be determined.

2) the synthesis of a series of isotopically labelled free acids and NAC thioesters of 9-hydroxynonanoic acid, and to carry out feeding studies with cultures of *Pseudomonas fluorescens*.

3) the preparation of isotopically labelled NAC thioesters of monic acid and its putative biosynthetic precursors, and to carry out feeding studies with cultures of *Pseudomonas fluorescens*.

Chapter 2

Synthetic routes to the proposed intermediates

2.1 Introduction

As discussed in chapter 1, previous biosynthetic studies have led to a hypothesis invoking the condensation of 9-hydroxynonanoic acid (69) and monic acid (50) to give pseudomonic acid (42). The work described in this chapter is focused on developing synthetic routes to a number of putative biosynthetic precursors to pseudomonic acid, required for feeding studies to test this hypothesis.

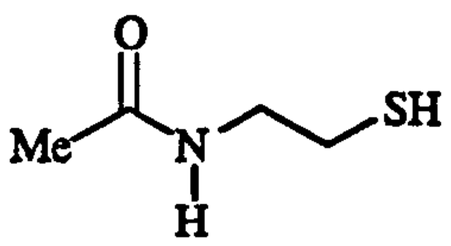
In terms of the biosynthesis of pseudomonic acid, these precursors fall into two distinct categories:

(i) those intermediates that may help to explain the origins of 9-hydroxynonanoic acid and,

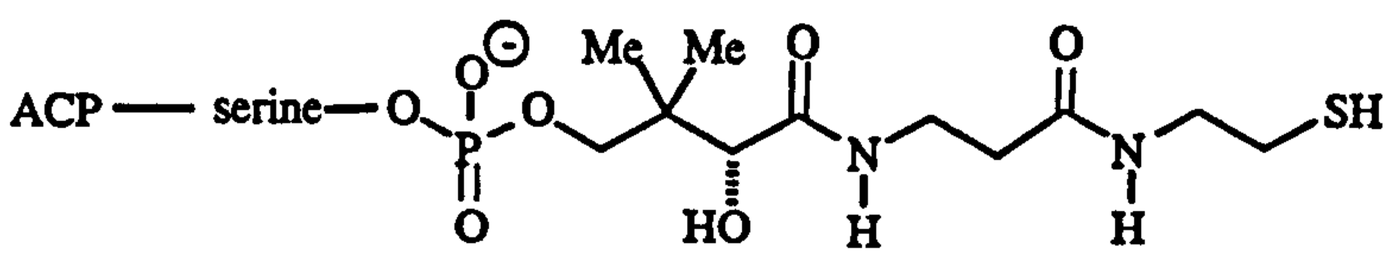
(ii) those intermediates required to elucidate the biosynthetic pathway to monic acid.

2.2 The value of N-acetylcysteamine thioesters in biosynthetic studies on fatty acids and polyketides

The use of N-acetylcysteamine thioesters (NAC thioesters) in biosynthetic studies was first demonstrated with studies on fatty acid biosynthesis.[78,79] It was suggested that the NAC thioester, obtained from N-acetylcysteamine (73), aids the transfer of the precursor onto the polyketide synthase (PKS) by mimicking the phosphopantotheine side chain of coenzyme A (75), and of the acyl carrier protein (ACP) component (74) of the PKS, which mediate the process *in vivo* (Fig. 2.1).

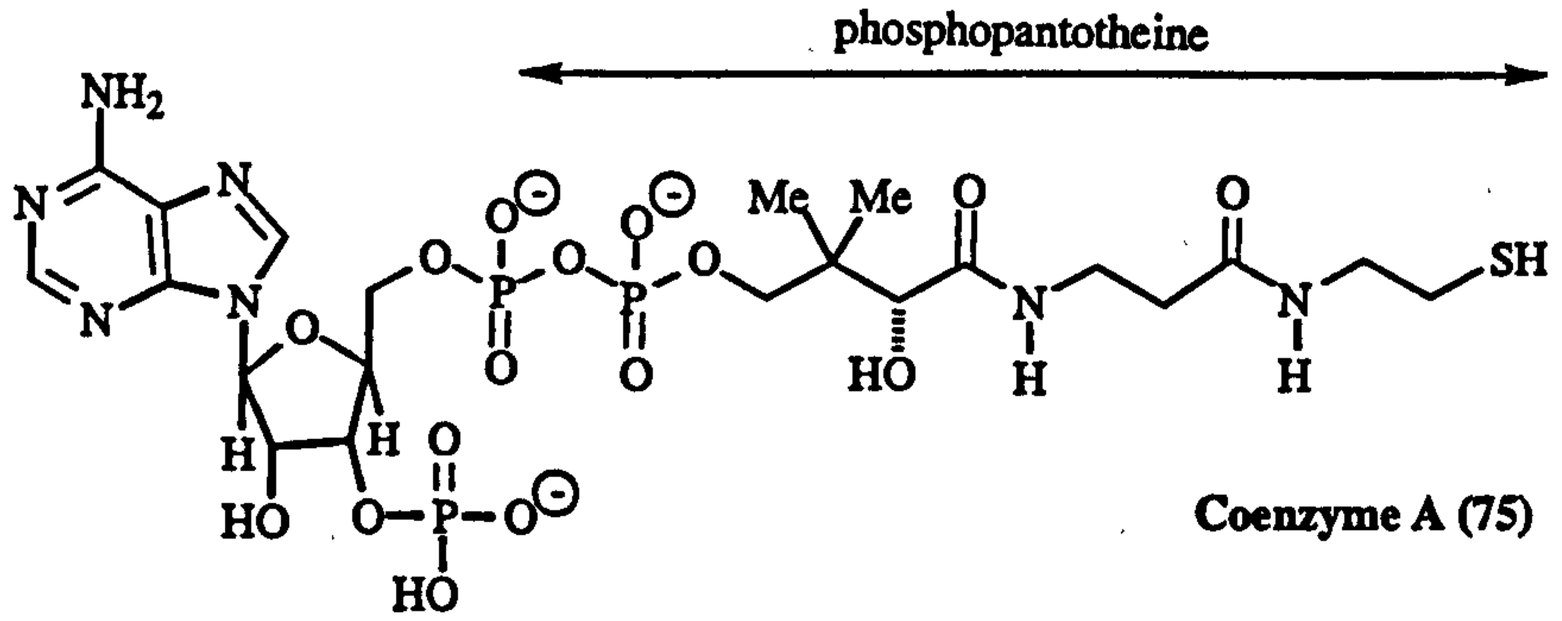


N-acetylcysteamine (73)



Acyl Carrier Protein (74)

phosphopantotheine



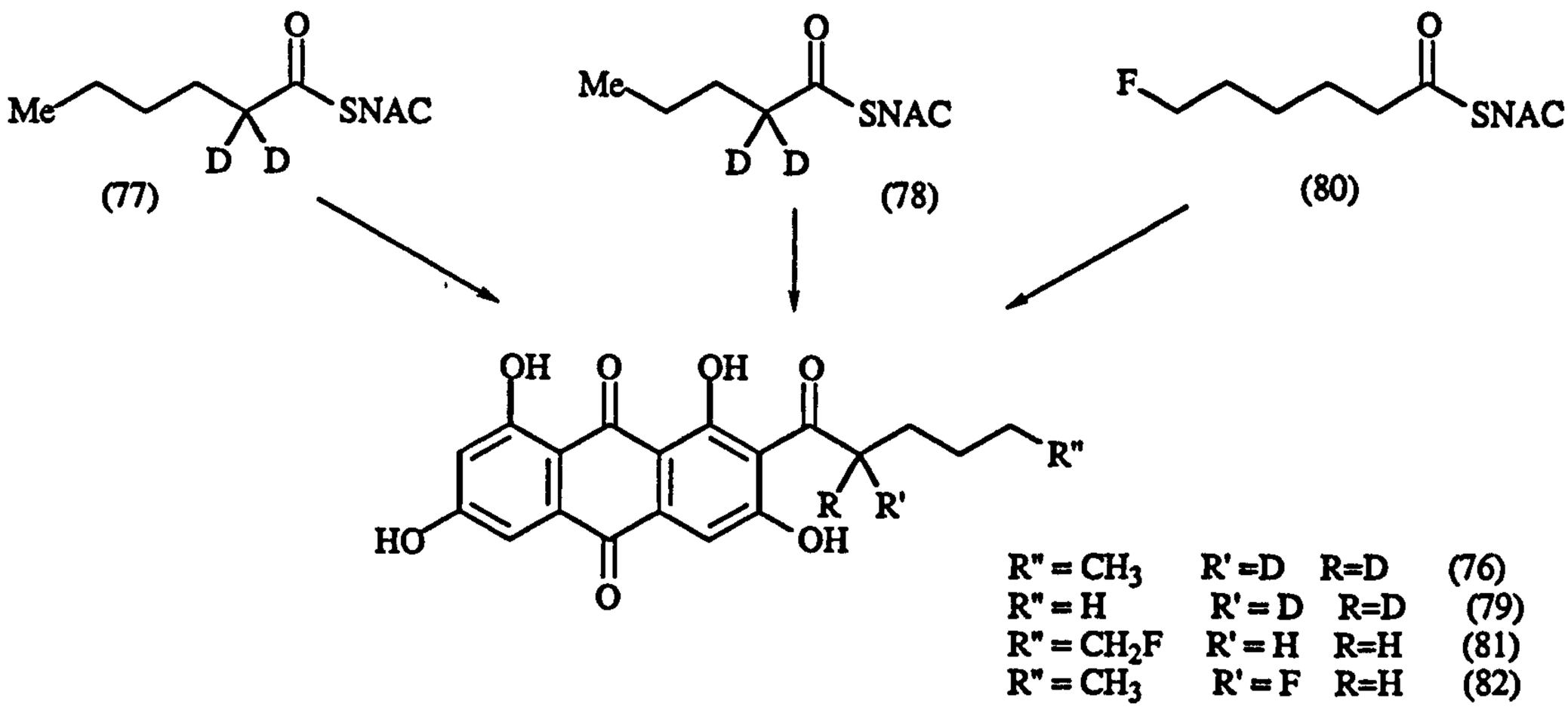
Coenzyme A (75)

Fig. 2.1: Structural similarities between N-acetylcytsteamine (NAC) (73), the ACP component of the PKS (74), and coenzyme A (75).

It has been demonstrated in many biosynthetic studies, such as those involving erythromycin B (7),[80] tylactone (26),[20] and aspyrone (40)[37] that feeding precursors in the form of the free acids leads to complete degradation before incorporation. Whereas when the precursors were administered as their N-acetylcysteamine thioesters (NAC thioesters), intact incorporation was observed.

Simpson and coworkers[81] recently reported that the intact incorporation of double deuterium labelled hexanoic acid into norsolorinic acid (76) is almost negligible. Whereas when administered as its NAC thioester (77), it was almost all incorporated intact, at a level of enrichment of 40%. Deuterium labelled ethyl hexanoate gave a lower level of enrichment, with a significant degree of incorporation via degradation.

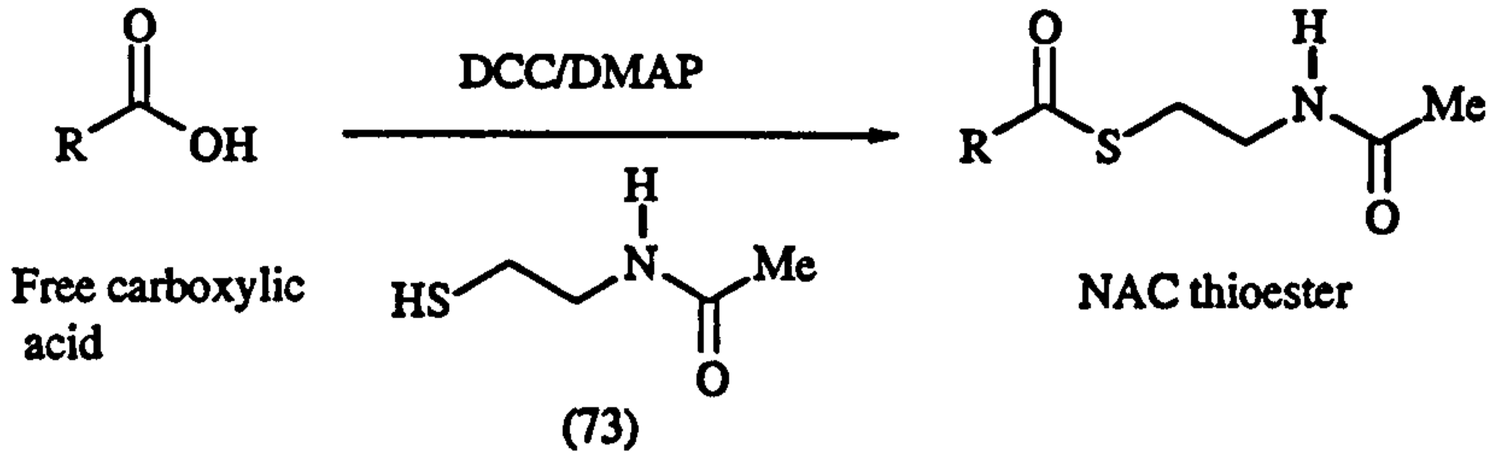
In the same paper the substrate specificity of the PKS, norsolorinic acid synthase (NSAS), was investigated by feeding deuterium labelled NAC thioesters of butyric acid, pentanoic acid, heptanoic acid, and octanoic acid to cultures of *Aspergillus parasiticus*. No incorporation of the deuterium label from butyrate, heptanoate, and octanoate was observed, whereas incorporation of the NAC thioester of the deuterium labelled pentanoic acid (78), was essentially identical to that observed with the hexanoate thioester, giving the corresponding analogue (79) of norsolorinic acid. Also, 6-fluorohexanoate (80) was synthesised and fed to cultures of *Aspergillus parasiticus*. Intact incorporation at a level of 36% was observed and a new metabolite (81) isolated. However, on feeding the corresponding 2-fluorohexanoate, there was no evidence of incorporation, and the analogue (82) of norsolorinic acid was not detected. This was possibly due to degradation, via β-oxidation, of 2-fluorohexanoate to the highly toxic 2-fluoroacetate, or that the 2-fluorohexanoate is a specific inhibitor of the NSAS.



Scheme 2.1: Incorporation of labelled starter units into norsolorinic acid (76), and their structural analogues.

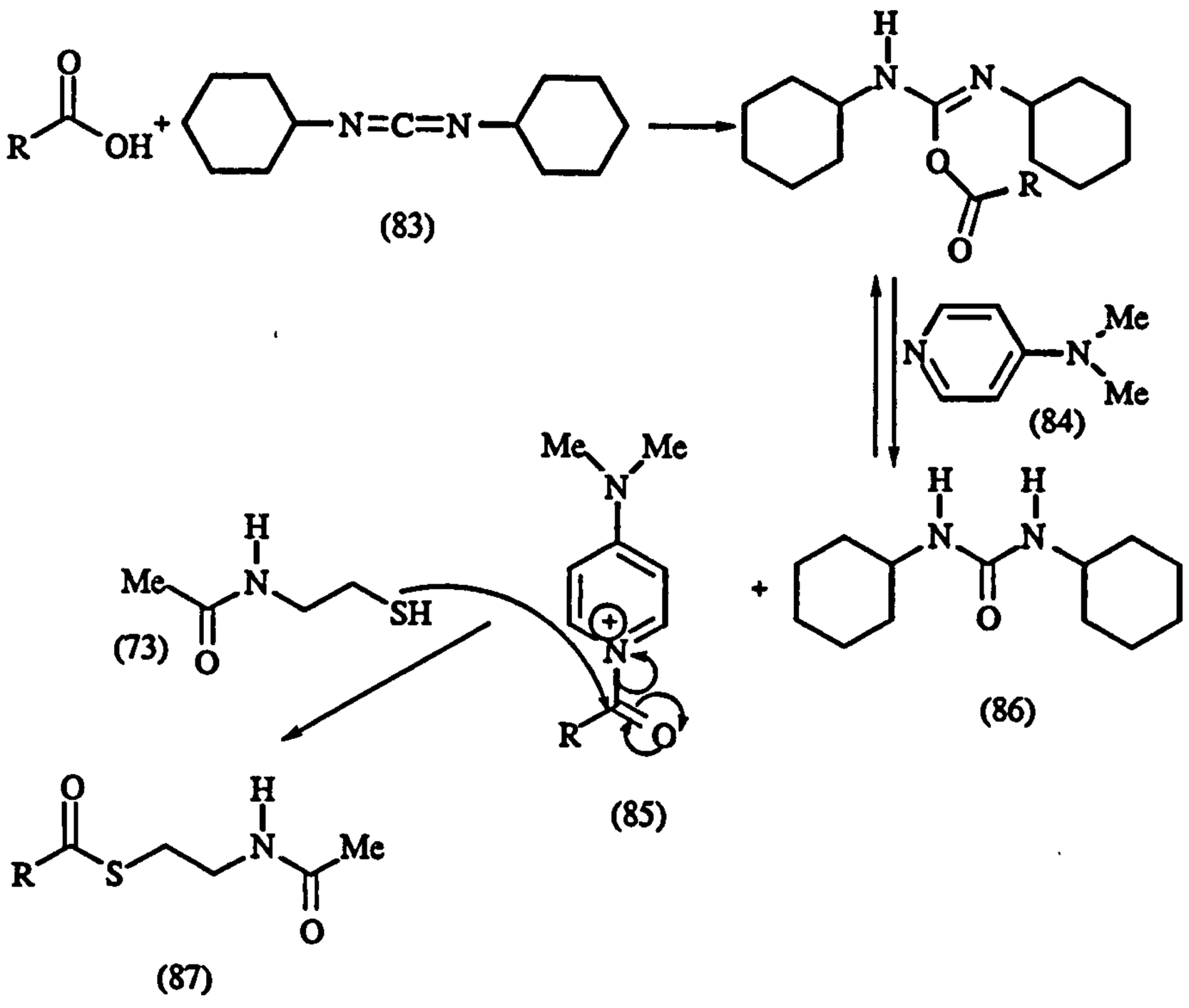
In addition, Westaway has shown that administering [1-^{13}C]benzoic acid and fluorinated benzoates as their NAC thioesters to the squalestatin producer *Phoma sp.* gives a greater level of incorporation compared to the free acid.[82]

In order to synthesise the NAC thioester from the free carboxylic acid, a condensation reaction between the free acid and N-acetylcysteamine (HSNAC) (73) must take place. The reaction is mediated by dicyclohexylcarbodiimide (DCC) (83), and is catalysed by 4,4-dimethylaminopyridine (DMAP) (84) (Scheme 2.2).[83]



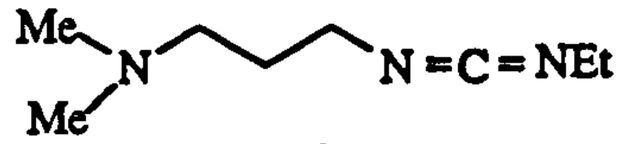
Scheme 2.2: Synthesis of NAC thioesters.

The postulated mechanism for a DCC/DMAP mediated thioesterification is shown below in Scheme 2.3.[84,85] The thioesterification is driven by the production of reactive N-acylpyridinium salts (85) and the formation of dicyclohexylurea (DCU) (86), which precipitates out of solution. After initial activation of the free acids by DCC, an equilibrium is set up between the activated carboxylic acid and the N-acylpyridinium salt. Nucleophilic attack of the thiol on the acyl group forms the required NAC thioester (87).



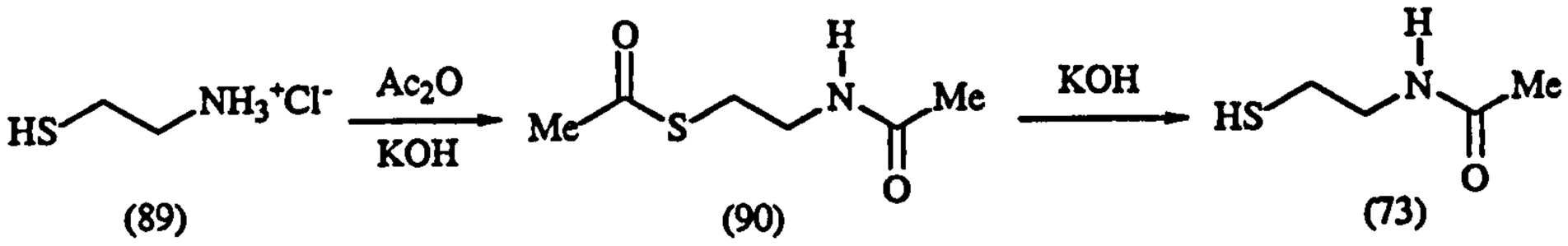
Scheme 2.3: Mechanism of the DCC/DMAP mediated synthesis of a thioester.

Removal of the precipitated DCU (86) is achieved by filtration through Celite, followed by flash column chromatography. It is often difficult to remove the last traces of DCU from the thioester, and this problem may be avoided by using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (88) rather than DCC. The corresponding urea can be removed, simply by washing with water.



(88)

N-acetylcysteamine may be prepared in high yield from readily available starting materials.[86] Acetylation of 2-mercaptoethylamine hydrochloride (89) gives N,S-diacetylcysteamine (90) in quantitative yield. Base hydrolysis of N,S-diacetylcysteamine forms N-acetylcysteamine in 92% yield (Scheme 2.4).



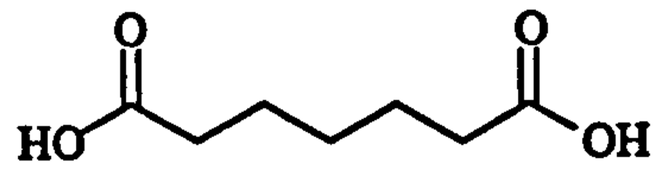
Scheme 2.4: Synthesis of N-acetylcysteamine.

For the majority of this work described in this chapter, the synthetic targets were the NAC thioesters of the corresponding free acids.

2.3 Investigation of the 9-hydroxynonanoic acid moiety

2.3.1 The biosynthesis of long chain hydroxy acids

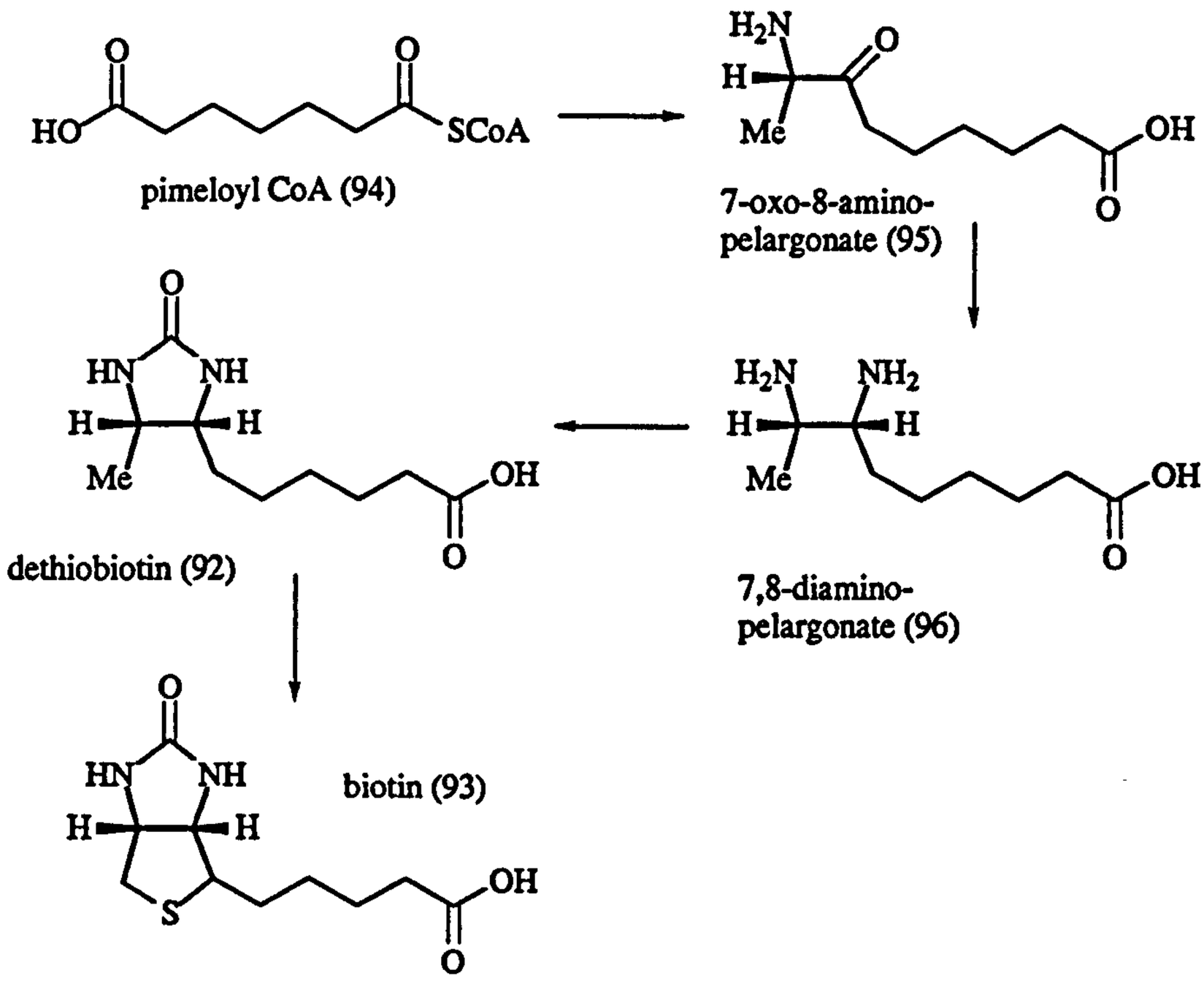
9-Hydroxynonanoic acid is a biosynthetically unique precursor. Reviewing the literature reveals a shortage of long chain dialkanoic acids involved in the biosynthesis of natural products. However, pimelic acid (91), or heptanedioic acid, has been found to be involved in biotin biosynthesis. Intact incorporation of [1-^{14}C]pimelic acid into dethiobiotin (92) and biotin (93) has led to pimeloyl CoA (94) being the earliest known precursor in the pathway of *de novo* biotin biosynthesis in *E. coli*.



(91)

The biosynthesis of biotin from pimeloyl CoA has been shown by Eisenberg,[87]

and Izumi and Ogata,[88] to proceed via the following intermediates, 7-oxo-8-amino-pelargonate (95), 7,8-diamino-pelargonate (96), and dethiobiotin (Scheme 2.5).



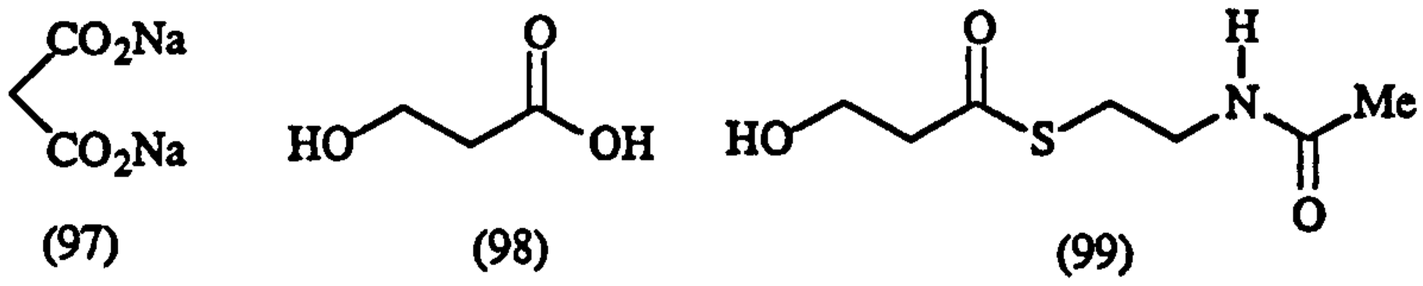
Scheme 2.5: Biosynthesis of biotin from pimeloyl CoA.

Lezius and coworkers[89] proposed that pimeloyl CoA is formed from the condensation of three molecules of malonyl CoA, on the basis of isotopic studies using *Achromobacter* grown in the presence of $^{14}CO_2$ with isovaleric acid as the main carbon source. These condensations of malonyl CoA can be explained as equivalent to normal fatty acid biosynthesis, but with a malonate starter unit. However, Ifuka and coworkers,[90] have shown that L-[3-^{13}C]alanine labelled the carbons of dethiobiotin in the same positions as that of D-[3-^{13}C]glucose. They inferred that alanine can be used as an alternative carbon source, and suggested that acetyl CoA (4) is a possible precursor for pimeloyl CoA. They also suggested that pimeloyl CoA may be biosynthesised from acetyl CoA, via methylation of caproyl CoA, which is derived from the normal fatty acid pathway.

This work did not further clarify the biosynthetic pathway of fatty acids with an odd number of carbon atoms in the chain. They concluded that pimeloyl CoA may be biosynthesised via the odd-numbered chain fatty acid metabolic pathway from propionyl CoA, or by ω-oxidation of odd-numbered chain fatty acid derived from the normal even-numbered chain fatty acid metabolic pathway.

2.3.2 Investigation of the starter units to 9-hydroxynonanoic acid

9-Hydroxynonanoic acid may be formed by 'normal' fatty acid type chain elongation of a C-3 starter unit, the origins of which are obscure. It was therefore necessary to develop synthetic routes to a number of possible C-3 starter units to 9-hydroxynonanoic acid. These include disodium malonate (97), 3-hydroxypropionic acid (98), and the NAC thioester (99) of 3-hydroxypropionic acid, in isotopically labelled form.



(97) (98) (99)

To investigate the role of malonate in pseudomonic acid biosynthesis, the synthesis of disodium malonate, via the route outlined in Scheme 2.6 was developed. Before proceeding with labelled material, the synthesis of disodium malonate was carried out on unlabelled material.

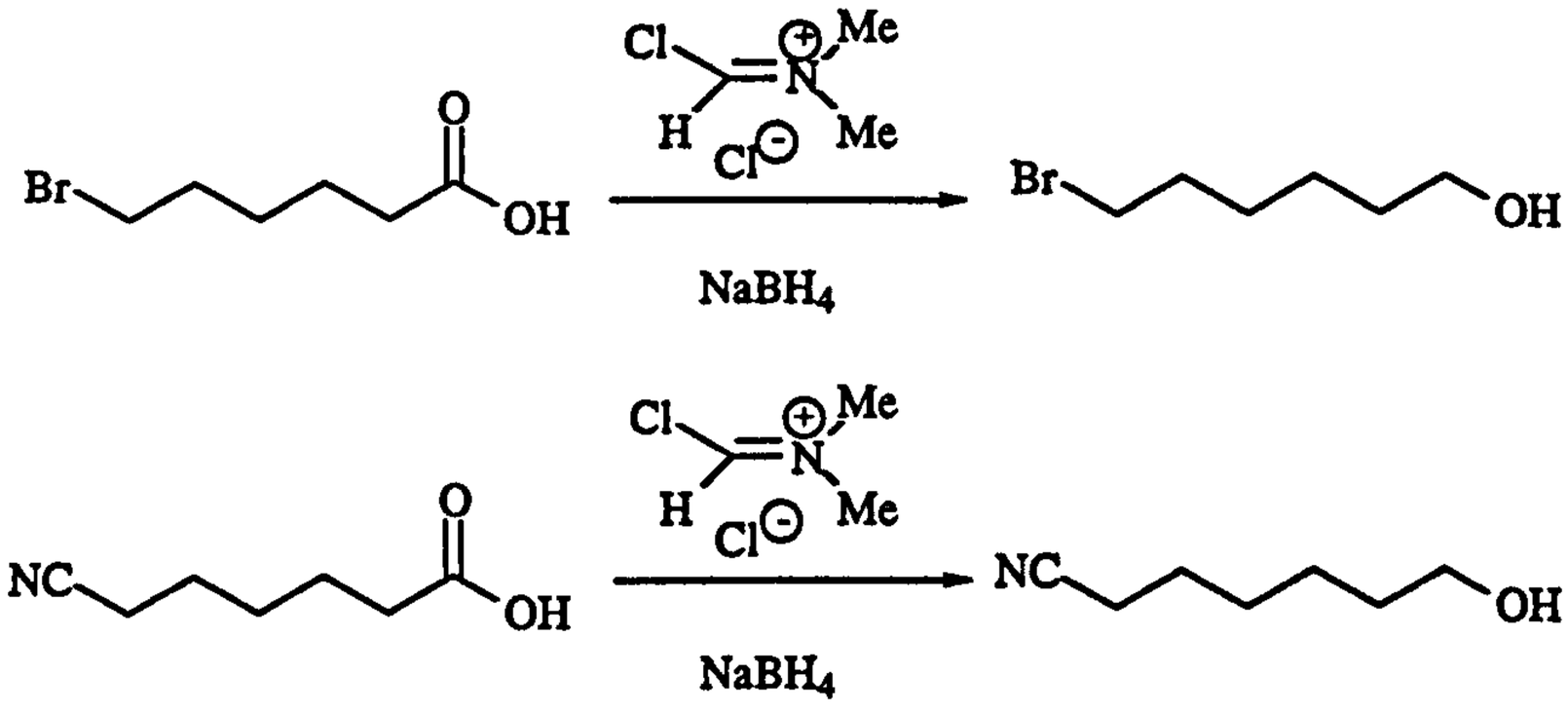
(100) (101) (102) (97)

Scheme 2.6: Synthesis of disodium malonate.

Based on a procedure reported by Ropp[91] in 1950, sodium acetate (100) was converted to ethyl acetate (101) in 70% yield, by heating under reflux with triethyl phosphate, and distillation into a liquid nitrogen trap. The lithium enolate was formed and reacted with ethyl chloroformate to produce diethyl malonate (102) in 57% yield. Saponification with sodium hydroxide of (102) gave disodium malonate (97) in 92% yield. The synthesis was then repeated, using sodium [1,2-$^{13}C_2$]acetate (103) as the source of isotopic labels giving disodium [1,2-$^{13}C_2$]malonate (104), via ethyl [1,2-$^{13}C_2$]acetate (105) and diethyl [1,2-$^{13}C_2$]malonate (106) with similar yields to those for unlabelled material.

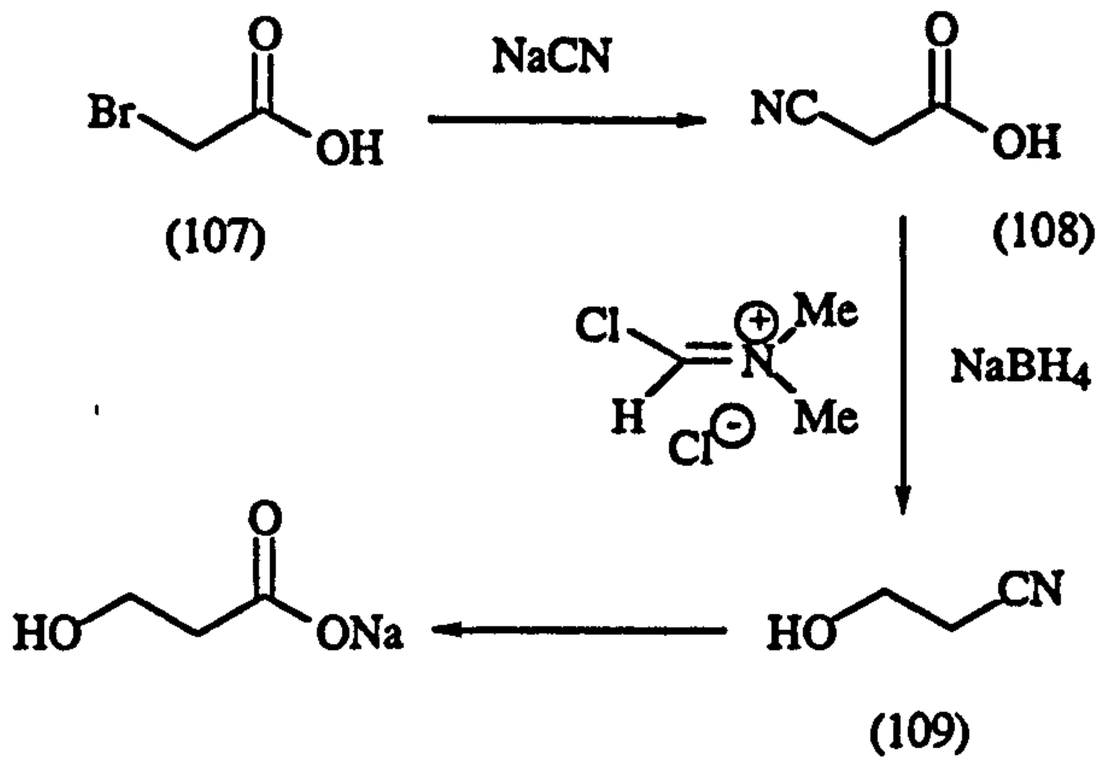
It is possible that 3-hydroxypropionic acid may be a starter unit, to 9-hydroxynonanoic acid, and that three fatty acid type chain elongations would produce the required C-9 moiety. Due to increased sensitivity in using a double $^{13}C_2$ label, as explained in section 1.2, a synthetic route to sodium [1,2-$^{13}C_2$]-3-hydroxypropionate was designed. Preliminary investigations have been carried out into the possible synthesis of sodium [1,2-$^{13}C_2$]-3-hydroxypropionate, using the procedure of Fujisawa and coworkers,[92] who reported the chemoselective reduction of a range of carboxylic acids

into alcohols, using N,N-dimethylchloromethyleniminium chloride and sodium borohydride. This is readily available from N,N-dimethylformamide and oxalyl chloride. Of particular interest was that they reported the chemoselective reduction of 6-bromohexanoic acid to 6-bromo-1-hexanol in 93%, and cyanohexanoic acid to cyanohexan-1-ol in 89%, as shown in Scheme 2.7.



Scheme 2.7: Chemoselective reduction of aliphatic carboxylic acids.

[2-^{13}C]Bromoacetic acid and [1-^{13}C]sodium cyanide are both commercially available, and were proposed as starting materials to [1,2-$^{13}C_2$]-3-hydroxypropionate (Scheme 2.8). Bromoacetic acid (107) was converted into cyanoacetic acid (108) by reaction with sodium cyanide in 82% yield, using the procedure of Lapworth and Baker.[93] The ^{1}H nmr spectrum was identical to that of an authentic sample.



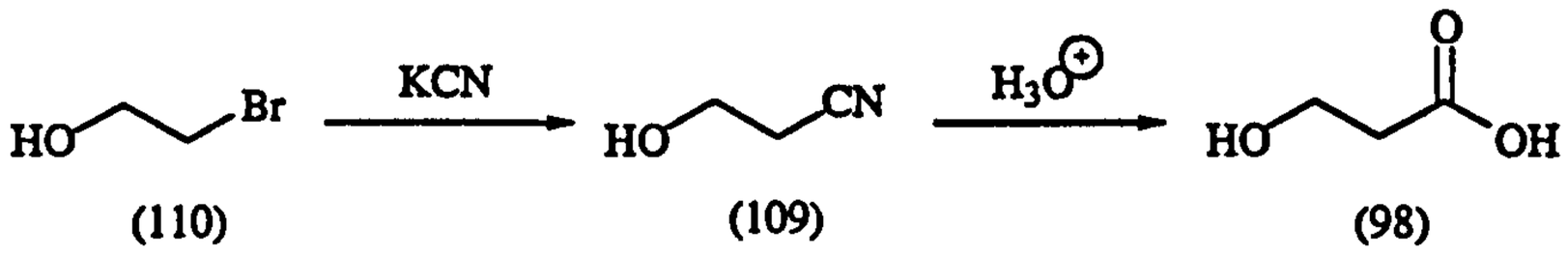
Scheme 2.8: Proposed synthesis of sodium 3-hydroxypropionate.

The chemoselective reduction of cyanoacetic acid was then attempted. The paper recommended the use of more than 2 equivalents of sodium borohydride to reduce aliphatic carboxylic acids. However, none of the conditions listed in Table 2.1 gave any of the required 3-hydroxypropionitrile (109).

Reaction Number	Amount of oxalyl chloride	Amount of cyano-acetic acid	Amount of $NaBH_4$	Temp. on addition of $NaBH_4$	Production of the hydroxynitrile (109)
1	3eq.	1eq.	5eq.	-30^0C	No
2	3eq.	1eq.	2eq.	-78^0C	No
3	3eq.	1eq.	2eq.	-30^0C	No

Table 2.1: Conditions used for the attempted chemoselective reduction of cyanoacetic acid

As an alternative, the route shown in Scheme 2.9 was proposed to synthesise isotopically labelled 3-hydroxypropionic acid, using readily available 2-bromoethanol (110) and potassium [1-^{13}C]cyanide, as the source of label introduction.

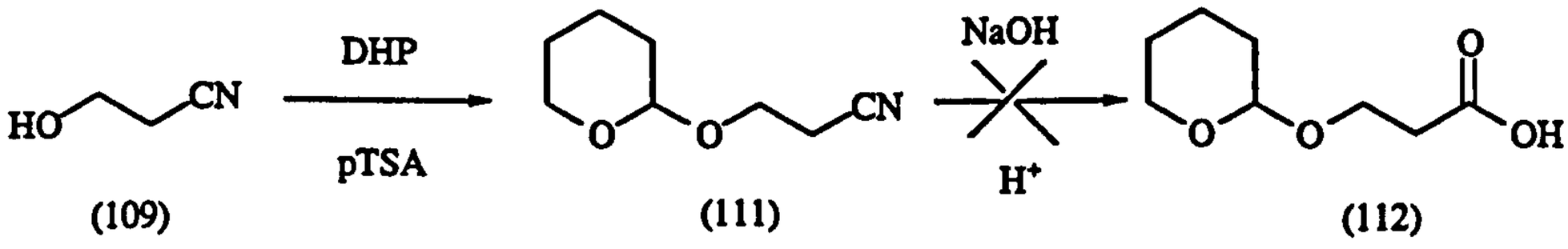


(110) (109) (98)

Scheme 2.9: Proposed synthesis of 3-hydroxypropionic acid (98).

A literature search showed that although the first step proceeded well under temperature controlled conditions,[94] the hydrolysis of the nitrile to 3-hydroxypropionic acid was low yielding.[95] This was indeed found to be the case. Reaction of 2-bromoethanol with potassium cyanide gave 3-hydroxypropionitrile (109) in 81% yield. The ^{1}H nmr spectrum was identical to that of an authentic sample. Base hydrolysis of the nitrile led to isolation of 3-hydroxypropionic acid in only 13% yield, probably due to 3-hydroxypropionic acid being very water soluble.

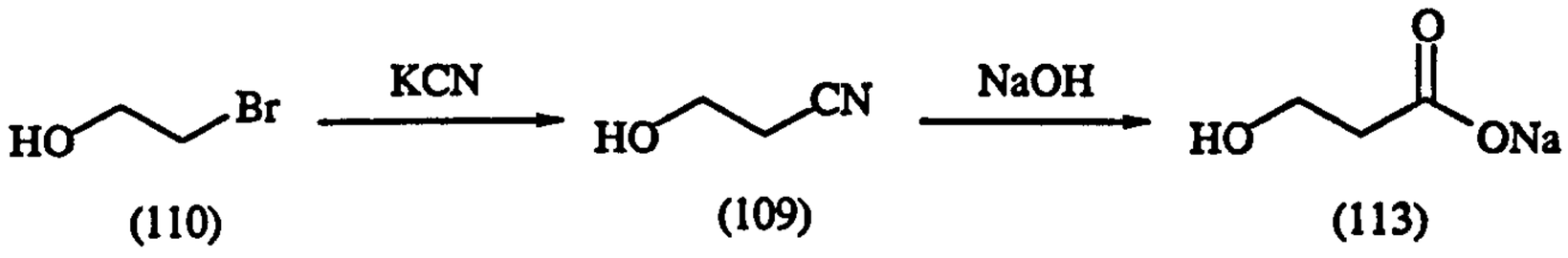
To overcome this problem of water solubility, 3-hydroxypropionitrile was protected as its THP ether (111), using dihydropyran and a catalytic amount of pTSA, in 72% yield. Attempted base hydrolysis of the protected nitrile gave a ^{1}H nmr spectrum, indicating a complex mixture of products, no signals of which corresponded to the required THP protected 3-hydroxypropionic acid (112) (Scheme 2.10).



(109) (111) (112)

Scheme 2.10: Attempted synthesis of the THP protected 3-hydroxypropionic acid.

The problem was finally overcome by synthesising the sodium salt (113) of 3-hydroxypropionic acid (Scheme 2.11).[96,97]



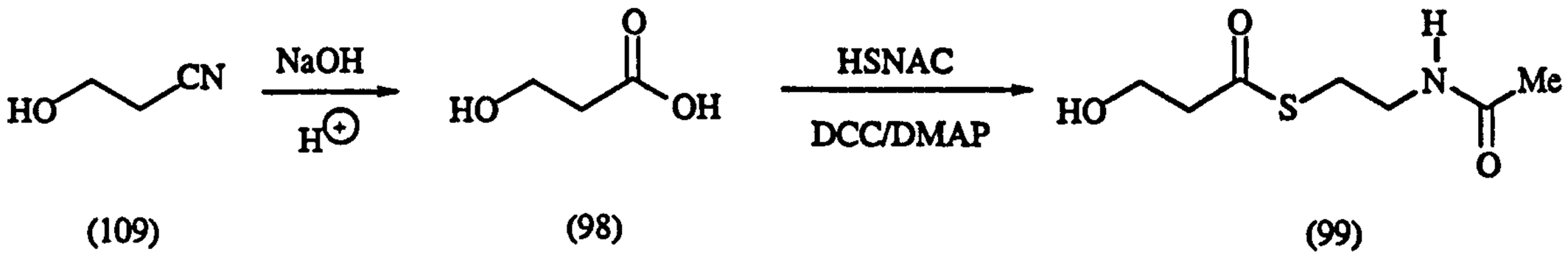
(110) (109) (113)

Scheme 2.11: Synthesis of sodium 3-hydroxypropionate.

3-Hydroxypropionitrile was prepared as before, and was hydrolysed with sodium hydroxide. Upon completion, the mixture was not acidified, and the aqueous extracts were freeze-dried to yield sodium 3-hydroxypropionate, as a crystalline solid in 95% yield.

As already discussed in section 2.2, the use of feeding putative labelled precursors as their NAC thioesters has been used to great effect to provide information on the biosynthetic pathways of a large number of polyketides. A synthesis of the NAC thioester of 3-hydroxypropionic acid (99) was therefore required.

The first attempt involved designing a route, suitable for a singly labelled version. The route outlined below was attempted (Scheme 2.12).

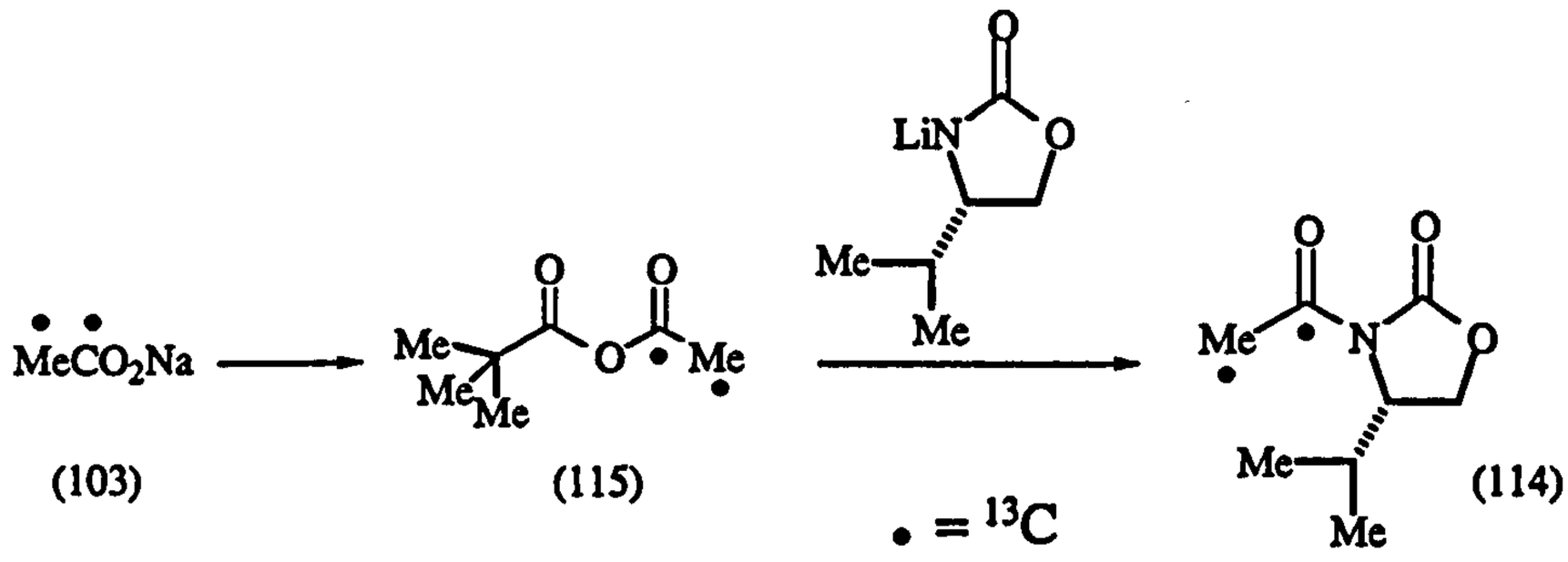


(109) (98) (99)

Scheme 2.12: Synthesis of the NAC thioester of 3-hydroxypropionic acid.

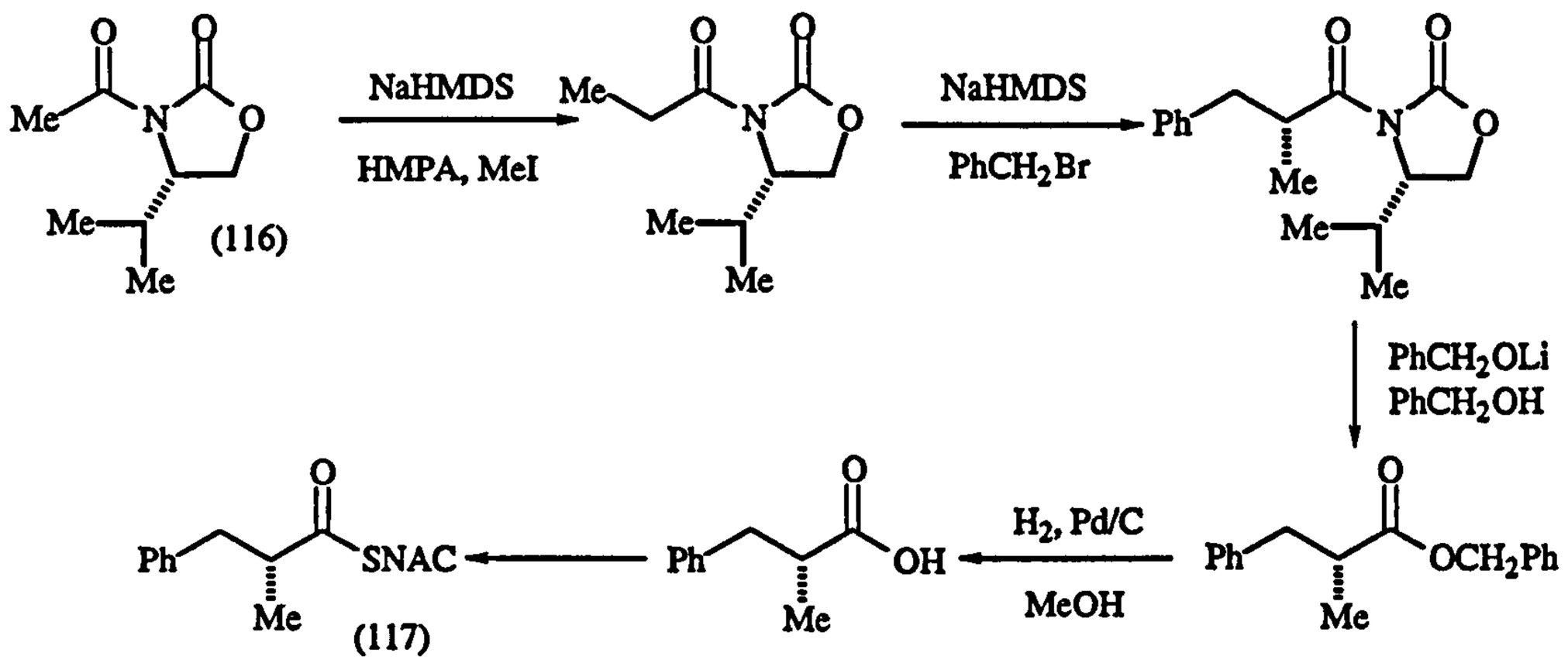
As discussed above, formation of 3-hydroxypropionic acid, via base hydrolysis of 3-hydroxypropionitrile, was achieved in only 13% yield.[95,98] The NAC thioester of 3-hydroxypropionic acid was prepared in 32% yield, using DCC/DMAP mediated coupling. Although the target product had been prepared, the overall yield of approximately 4% yield over the final 2 steps was unacceptable for the synthesis of the NAC thioester of [1-^{13}C]-3-hydroxypropionic acid to be carried out. Other possible routes were therefore investigated.

(4*S*)-[1,2-$^{13}C_2$]-3-Acetyl-4-(1-methylethyl)-2-oxazolidinone (114) had been prepared previously from sodium [1,2-$^{13}C_2$]acetate, via the mixed anhydride of trimethylacetyl chloride (115) (Scheme 2.13).[82]



Scheme 2.13: Synthesis of (4*S*)-[1,2-$^{13}C_2$]-3-acetyl-4-(1-methylethyl)-2-oxazolidinone.

The N-acetyloxazolidinone (116) has proved to be a valuable intermediate in the synthesis of a range of isotopically labelled precursors to polyketides, *e.g.* the NAC thioester of [1,2-$^{13}C_2$]-(2*R*)-benzyl propionic acid (117) (Scheme 2.14).[82]

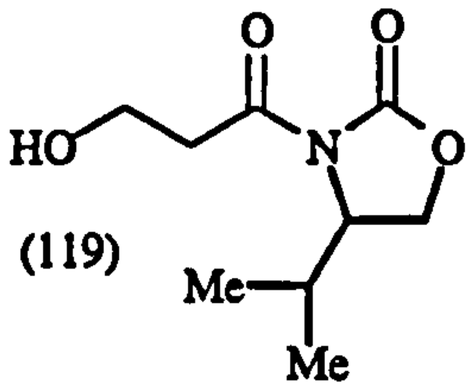


Scheme 2.14: Synthesis of the NAC thioester of (2*R*)-benzyl propionic acid.

Westaway[82] has also shown that by forming the dianion of N-acetylcysteamine with 2 equivalents of sodium hydride, it is possible to directly displace the chiral auxiliary to give an NAC thioester, *e.g.* in the synthesis of the β-keto NAC thioester (118) (Scheme 2.15).

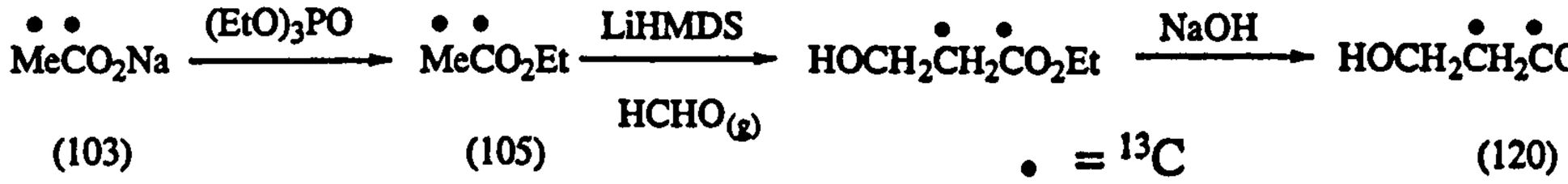
Scheme 2.15: Synthesis of the β-keto NAC thioester via direct displacement of the chiral auxiliary.

Although, we are not introducing any stereocentres in the NAC thioester of 3-hydroxypropionic acid (99), an oxazolidinone would be a useful handle, not only to reduce the volatility of these small molecules, but also to reduce their water solubility. The 2-oxazolidinone can readily be removed, and recovered for reuse. By directly displacing the 2-oxazolidinone with N-acetylcysteamine, formation of the free acid is avoided. In order to utilise the direct displacement reaction, in the synthesis of the NAC thioester of [1,2-$^{13}C_2$]-3-hydroxypropionic acid, our intermediate target was the 3-hydroxypropionyl-4-(1-methylethyl)-2-oxazolidinone (119)



(119)

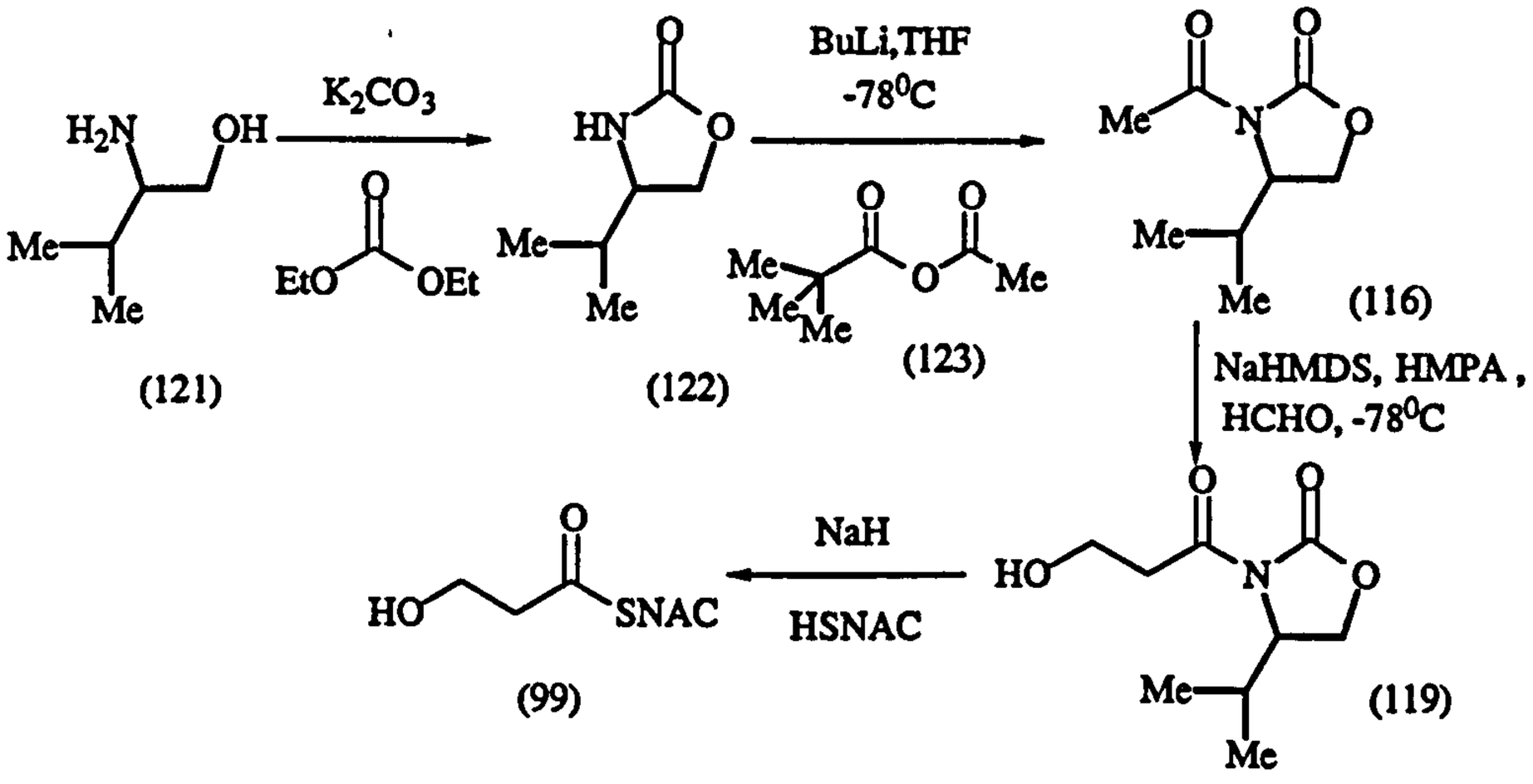
In 1985, Gutman and Ribon[99] reported the formation of sodium [1,2-$^{13}C_2$]-3-hydroxypropionate (120), starting from sodium [1,2-$^{13}C_2$]acetate (103) (Scheme 2.16). The key step was the reaction of ethyl acetate with gaseous formaldehyde, formed by heating the paraformaldehyde trimer, and passing the gas in a steady stream of nitrogen.



(103) (105) (120)

Scheme 2.16: Synthesis of sodium [1,2-$^{13}C_2$]-3-hydroxypropionate

An analogous procedure was used in the hydroxymethylation of the acetyloxazolidinone (116) (Scheme 2.17).



(121) (122) (123) (116) (119) (99)

Scheme 2.17: Proposed synthesis of the NAC thioester of 3-hydroxypropionic acid.

This approach also allows the introduction of a double $^{13}C_2$ label at the N-acylation stage. The oxazolidinone (122) was prepared by heating to reflux (*RS*)-valinol (121) and diethyl carbonate in 66% yield.[100] N-acylation of the auxiliary gave 3-acetyl-4-(1-methylethyl)-2-oxazolidonone (116) in 84% yield, via the sodium enolate.

Hydroxymethylation of the sodium enolate of the N-acetyl oxazolidonone was examined using two different methods to generate formaldehyde.

Method 1

By heating flask 1 (Fig. 2.2) to 150 °C the paraformaldehyde trimer was monomerised and an excess of formaldehyde gas transferred into flask 2 (by pressure, via a length of PTFE tubing) to saturate the preformed sodium enolate of the N-acetyl oxazolidinone. The reaction mixture was allowed to stir at -78 °C for three hours after which work up yielded a colourless oil.

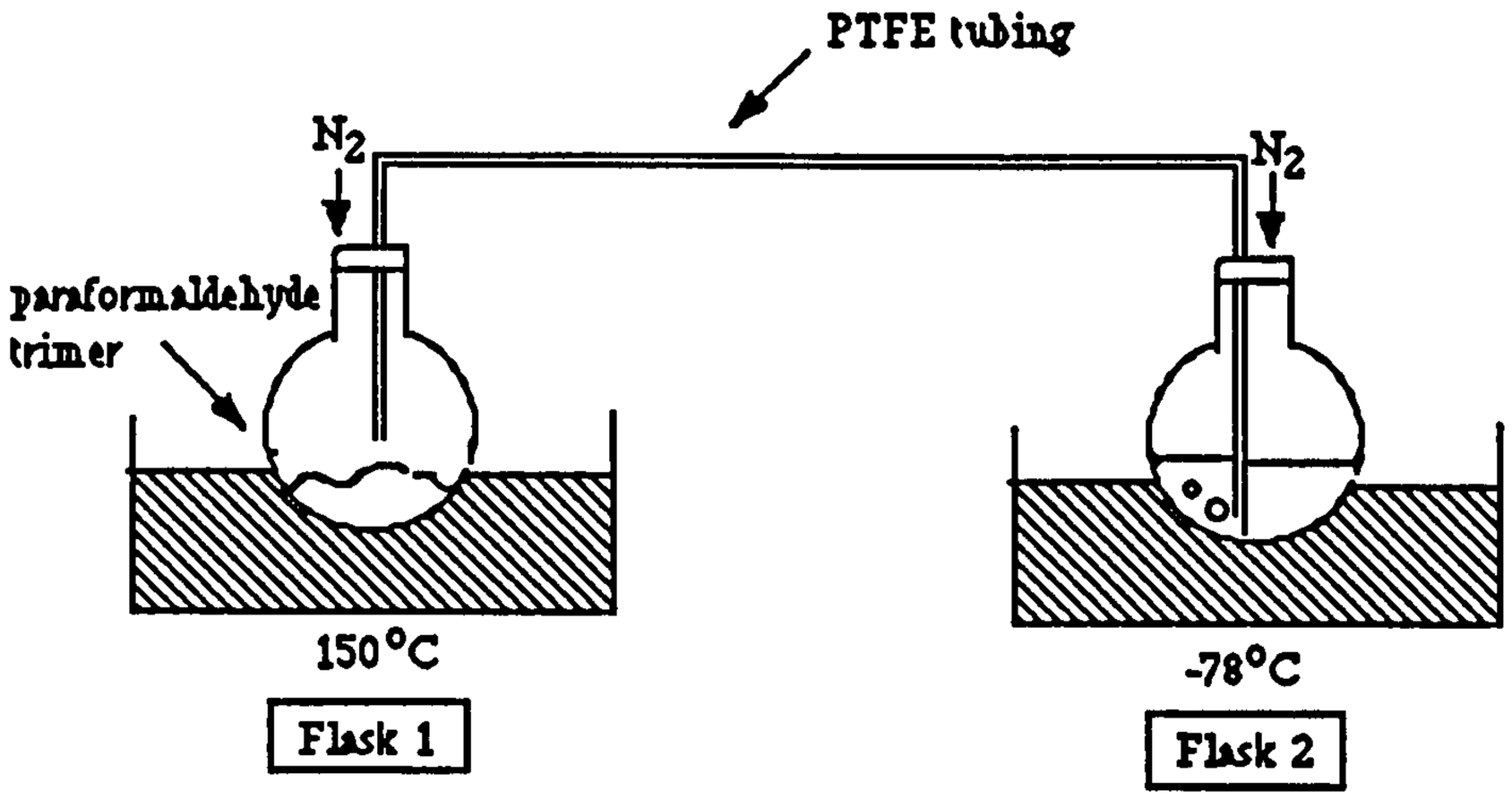
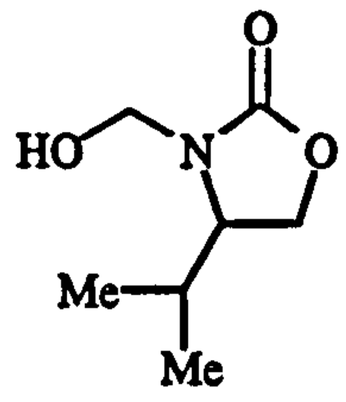


Fig. 2.2: Apparatus used for attempted formylation of the N-acetyloxazolidnone.

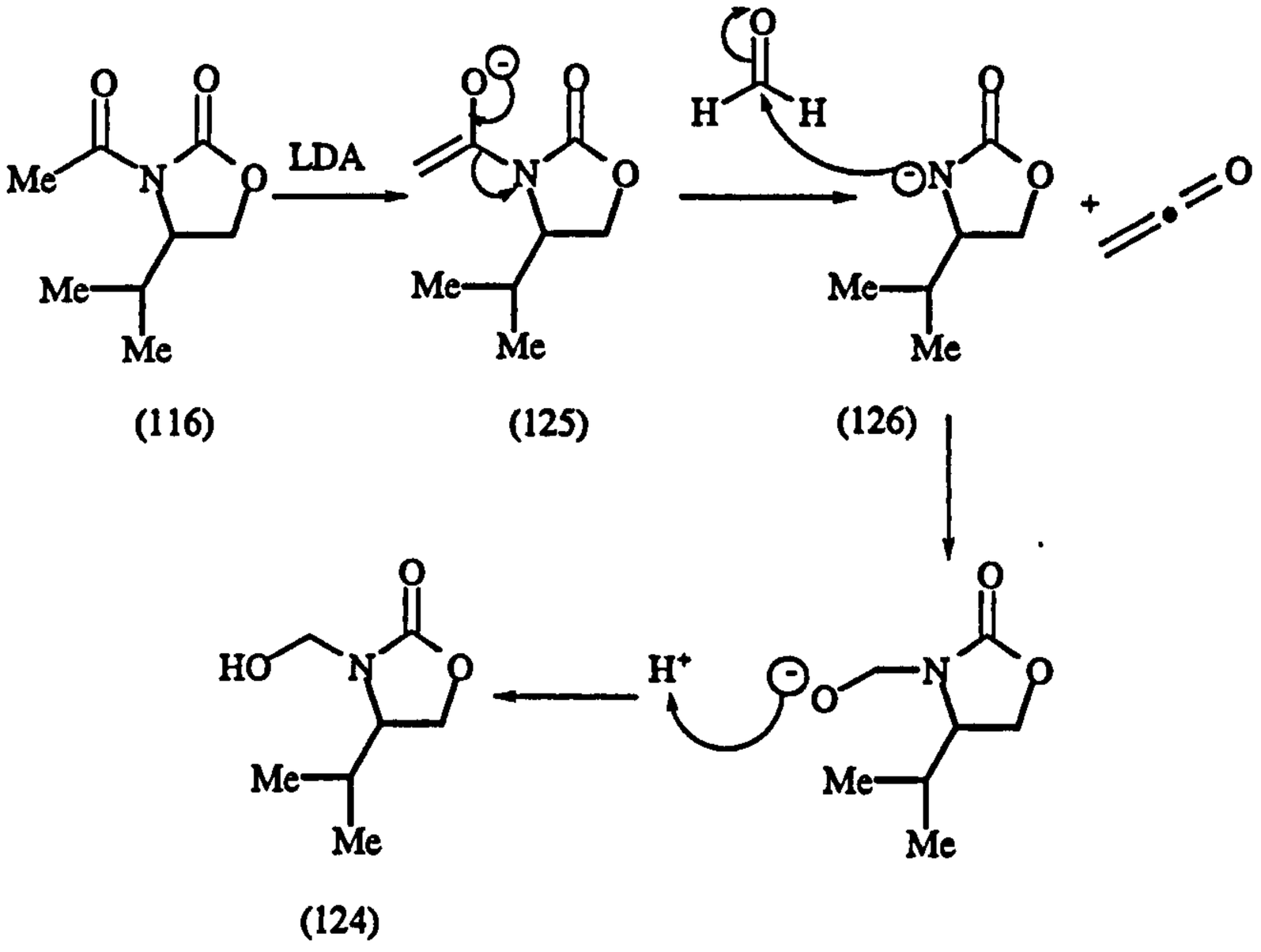
The ^1H nmr spectrum revealed that the desired product, 3-hydroxypropionyl-4-(1-methylethyl)-2-oxazolidinone (119) had not been formed, but that 3-hydroxymethyl-4-(1-methylethyl)-2-oxazolidinone (124) had been obtained in 83% yield. There was some evidence by TLC for the return of some oxazolidinone (122).

(124)



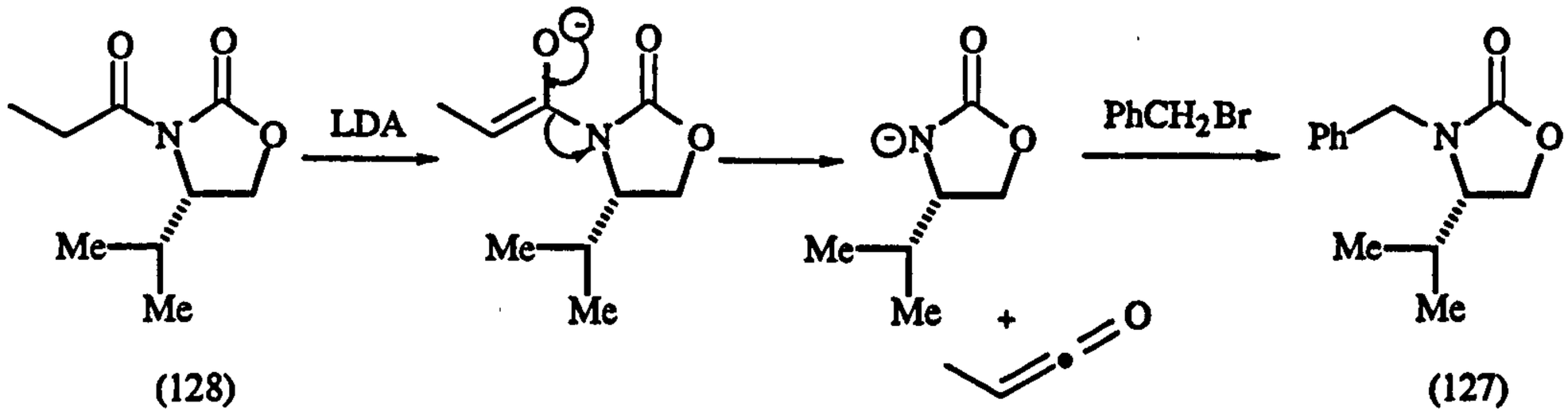
The ^1H nmr spectrum of (124) showed an upfield shift of the oxazolidinone

signals from δ 2.40, 4,24, and 4.43 to δ 2.15, 4.01, and 2.28 respectively, due to the loss of the electron withdrawing (deshielding) effect of the N-acetyl group. In addition the singlet at δ 2.54, assigned to CH_3CO, had disappeared, but two doublets at δ 4.68 and 4.95 (each showing coupling of *J* 11.6Hz) were apparent, and assigned to the hydroxymethyl group. The mass spectrum showed the expected molecular ion of 159. The formation of (124) may be rationalised by the decomposition of the sodium enolate (125) to give the anion of the oxazolidinone (126), which was subsequently reacted with the added electrophile, formaldehyde (Scheme 2.18).



Scheme 2.18: Proposed mechanism for the formation of (124).

It is known that as the 2-oxazolidinone is a good leaving group, and that the enolate can decompose via a ketene pathway (Scheme 2.18).[101] Similar observations have been reported by Westaway,[82] who obtained the benzyl oxazolidinone (127) on reaction of N-propionyloxazolidinone (128) with benzyl bromide, as shown in Scheme 2.19.



Scheme 2.19: Reaction of N-propionyl oxazolidinone with benzyl bromide.

Evans[101] reported that lithium enolates decompose at temperatures above 0 °C, but Westaway observed decomposition above -60 °C. Although the reaction mixture in method 1 was maintained at -78 °C, it was thought that the formaldehyde gas introduced into the reaction flask 2 via the PTFE tubing could possibly have created pockets of higher temperature. Thus method 2 was attempted to maintain consistently low temperatures of -78 °C while adding the formaldehyde to the reaction mixture.

Method 2

The apparatus shown in Figure 2.3 was designed for effective transfer of formaldehyde to the pre-formed anion at -78 °C. A saturated solution of formaldehyde in THF was prepared by heating anhydrous paraformaldehyde to 150 °C, and the formaldehyde gas produced was transferred via a length of PTFE tubing into a flask containing anhydrous THF. The saturated solution was then transferred to flask 1, as shown in Fig. 2.4, which was then added to the preformed anion of the N-acetyl oxazolidinone in flask 2 by carefully tipping the apparatus, while at -78 °C. This method ensured the temperature of the reaction did not increase above -78 °C.

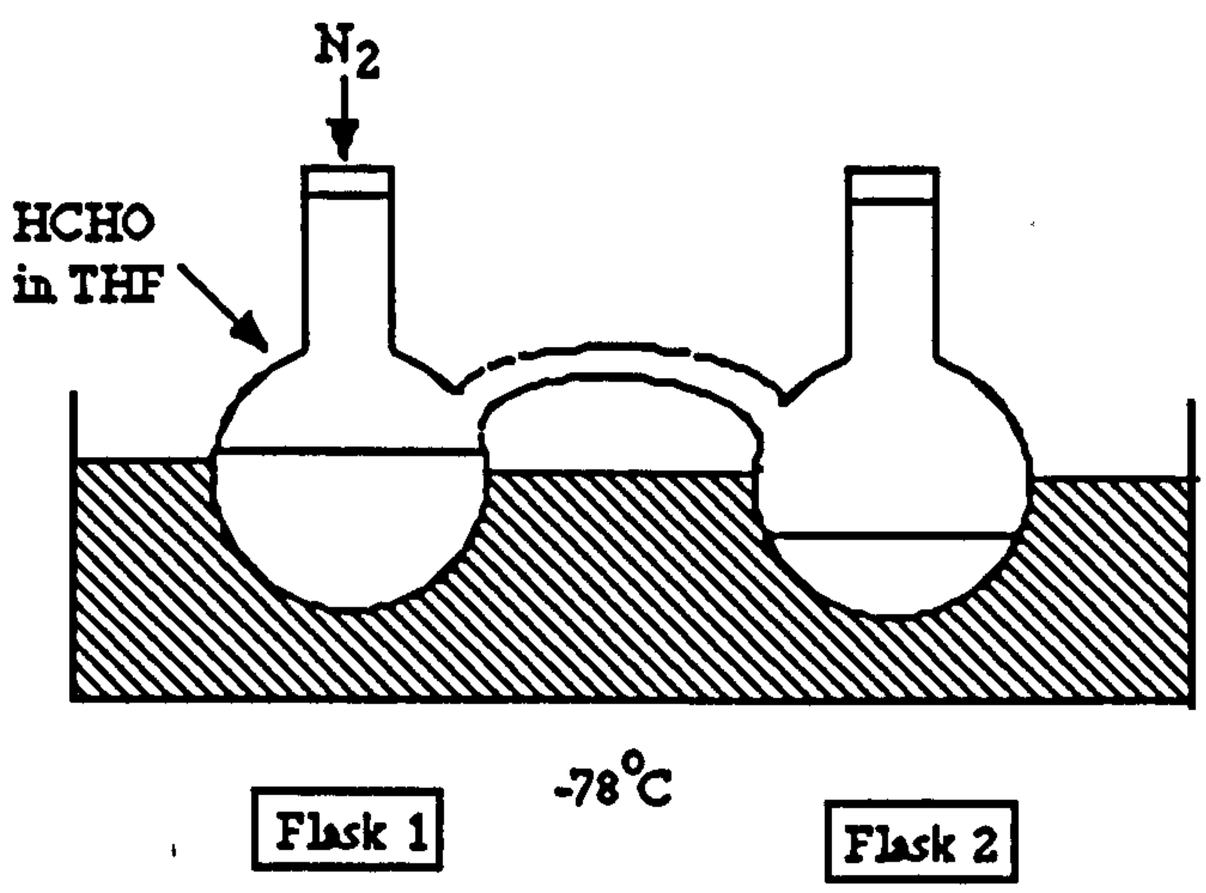
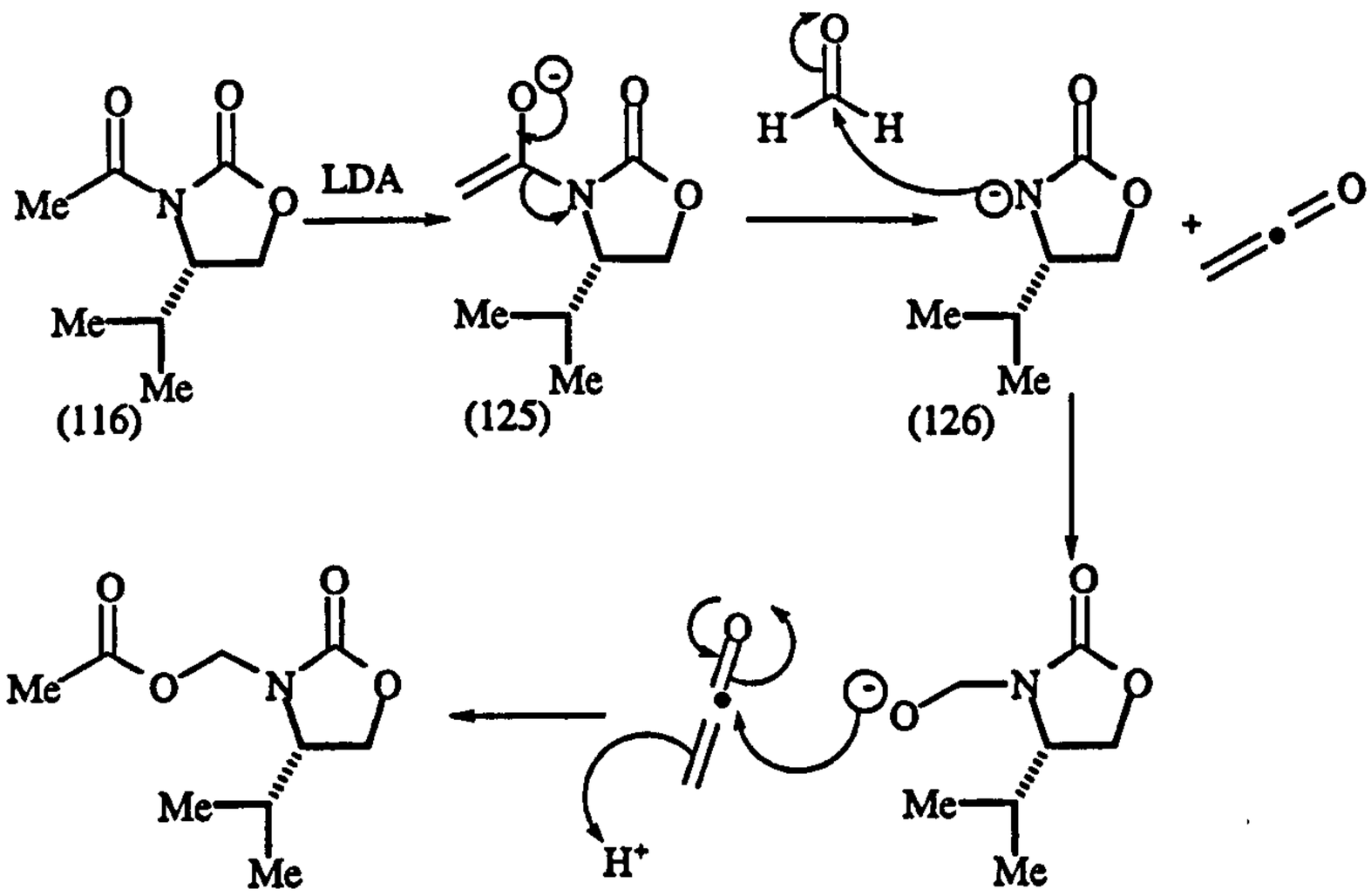


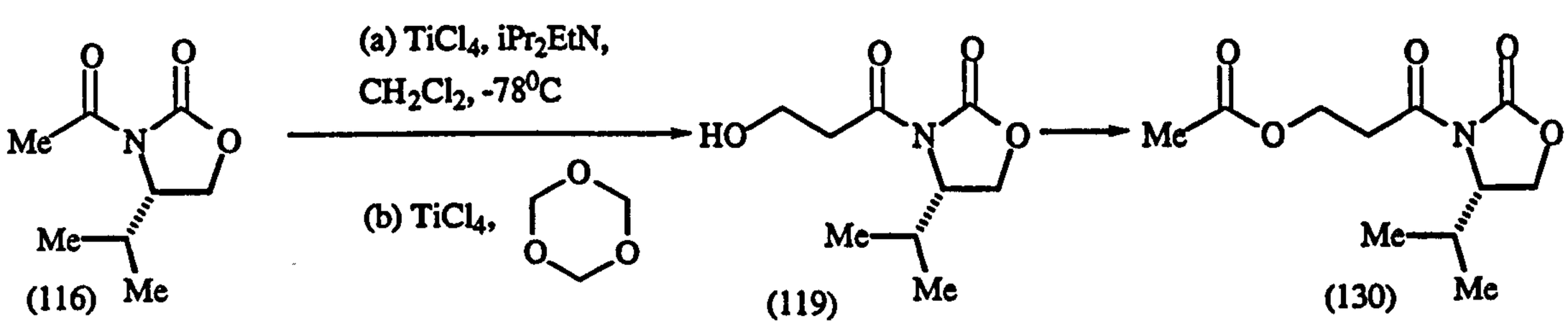
Fig. 2.3: Apparatus for transfer of a saturated THF with formaldehyde to the anion at -78 °C.

However, again 3-hydroxymethyl-(1-methylethyl)-2-oxazolidinone (124) was the sole product isolated from the reaction mixture. Miller, in our laboratories, experienced similar difficulties in synthesising (4*S*)-3-hydroxypropionyl-4-(1-methylethyl)-2-oxazolidinone (119), using these routes.[102] However, she obtained (4*S*)-3-acetoxy-4-(1-methylethyl)-2-oxazolidinone (129) as the sole product from the reaction. Esters may therefore be formed (Scheme 2.20).



Scheme 2.20: Proposed mechanism for the formation of (129).

Recently, Watts[103] has reported the successful hydroxymethylation, by reacting the titanium enolate of the N-acetylyloxazolidinone (116) with trioxane, in the presence of titanium tetrachloride ($TiCl_4$) (Scheme 2.21).[104]



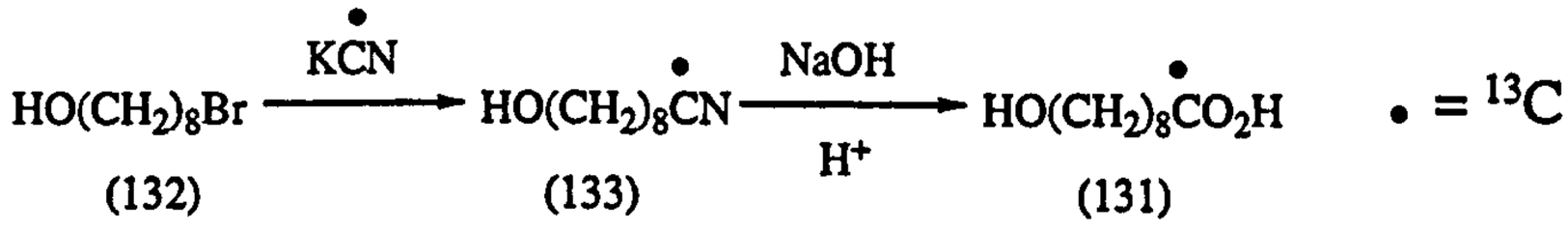
Scheme 2.21: Synthesis of 3-hydroxypropionyl-4-(1-methylethyl)-2-oxazolidinone.

On purification by column chromatography, the alcohol (119) decomposed. However, acetylation of (119) under standard conditions gave the acetate (130), which was stable to chromatography.

Work towards the direct displacement of the chiral auxiliary, using a derivative of (119) with a protected alcohol, by N-acetylcysteamine to give the NAC thioester of 3-hydroxypropionic acid is currently being examined by Watts.

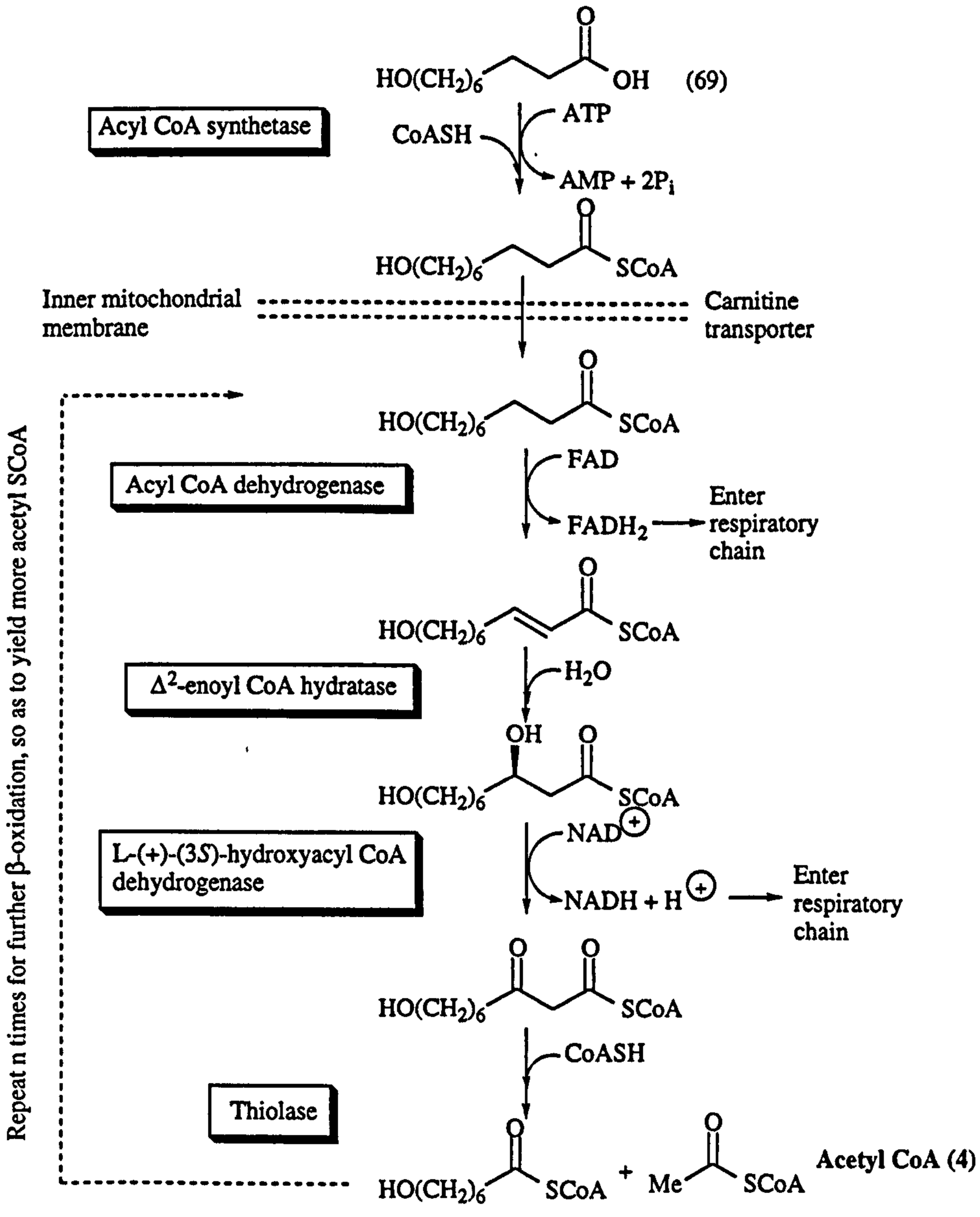
2.3.3 Synthesis of isotopically labelled 9-hydroxynonanoic acid

Sugden[77] has prepared [1-^{13}C]-9-hydroxynonanoic acid (131), as shown in Scheme 2.22.



Scheme 2.22 Synthesis of [1-^{13}C]-9-hydroxynonanoic acid.

On addition of [1-^{13}C]-9-hydroxynonanoic acid to *Pseudomonas fluorescens* NCIB 10586, following isolation of the pseudomonic acid, he found that the label had been efficiently incorporated into pseudomonic acid, but that it was incorporated non-specifically. Degradation to acetate, through β-oxidation, had occurred prior to incorporation into pseudomonic acid.[77] The process of β-oxidation is outlined in Scheme 2.23.



Scheme 2.23: β-Oxidation of 9-hydroxynonanoic acid.

A number of inhibitors to fatty acid catabolic enzymes have been developed, of which one of the most effective is tetradecylthiopropionic acid (134).[105]

$CH_3(CH_2)_{13}SCH_2CH_2CO_2I$

(134)

On feeding [1-^{13}C]-9-hydroxynonanoic acid along with tetradecylthiopropionic acid to *Pseudomonas fluorescens*, Sugden again found efficient non-specific incorporation of isotopic label.[77] Due to the result of competitive catabolism β-oxidation, no firm conclusions could be made from these series of experiments.

It has previously been shown that feeding NAC thioesters, rather than the free acid, overcomes the problem associated with intact incorporation (see section 2.2). Therefore, our target molecules were the NAC thioesters of [1,2-$^{13}C_2$] and [2,3-$^{13}C_2$]-9-hydroxynonanoic acid. The need for both patterns of labelling are as follows.

Firstly, as catabolism had already been shown to be a dominant pathway, the labels needed to be present such that any intact incorporation could be observed. It may therefore be expected that β-oxidation of 9-hydroxynonanoic acid may cause cleavage of acetate units from the C-1 end of the molecule, as shown in Fig. 2.4. It is therefore necessary to synthesise the NAC thioester of [2,3-$^{13}C_2$]-9-hydroxynonanoic acid, so that we can discriminate, in terms of coupling, the difference between intact incorporation and degradation to acetate.

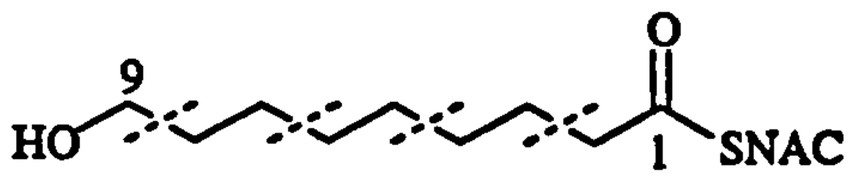


Fig. 2.4: Possible cleavage of acetate during β-oxidation of 9-hydroxynonanoic acid.

On the other hand, it is not known whether there may be a competing biological oxidation at C-9 followed by cleavage of acetate units from this end of the molecule, as outlined in Fig. 2.5. For the same reasons given above, it is therefore necessary to synthesise the NAC thioester of [1,2-$^{13}C_2$]-9-hydroxynonanoic acid in order to probe this possible oxidation process.

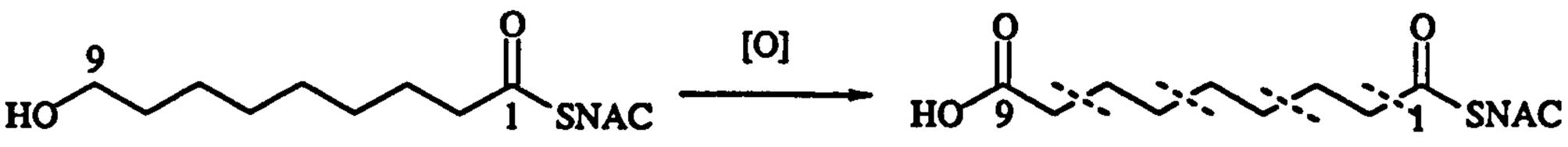


Fig. 2.5: Possible cleavage of acetate during β-oxidation of 9-hydroxynonanoic acid.

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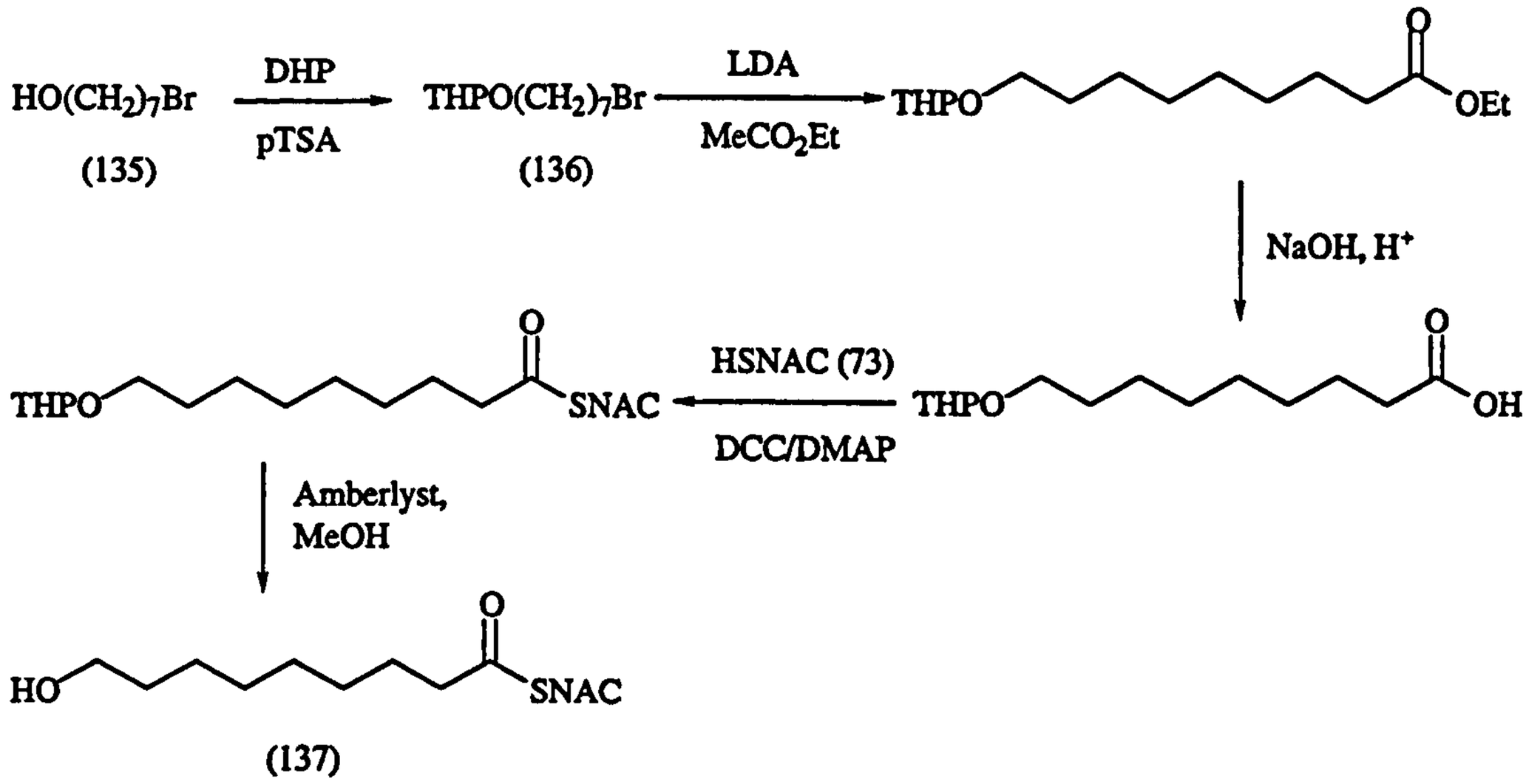
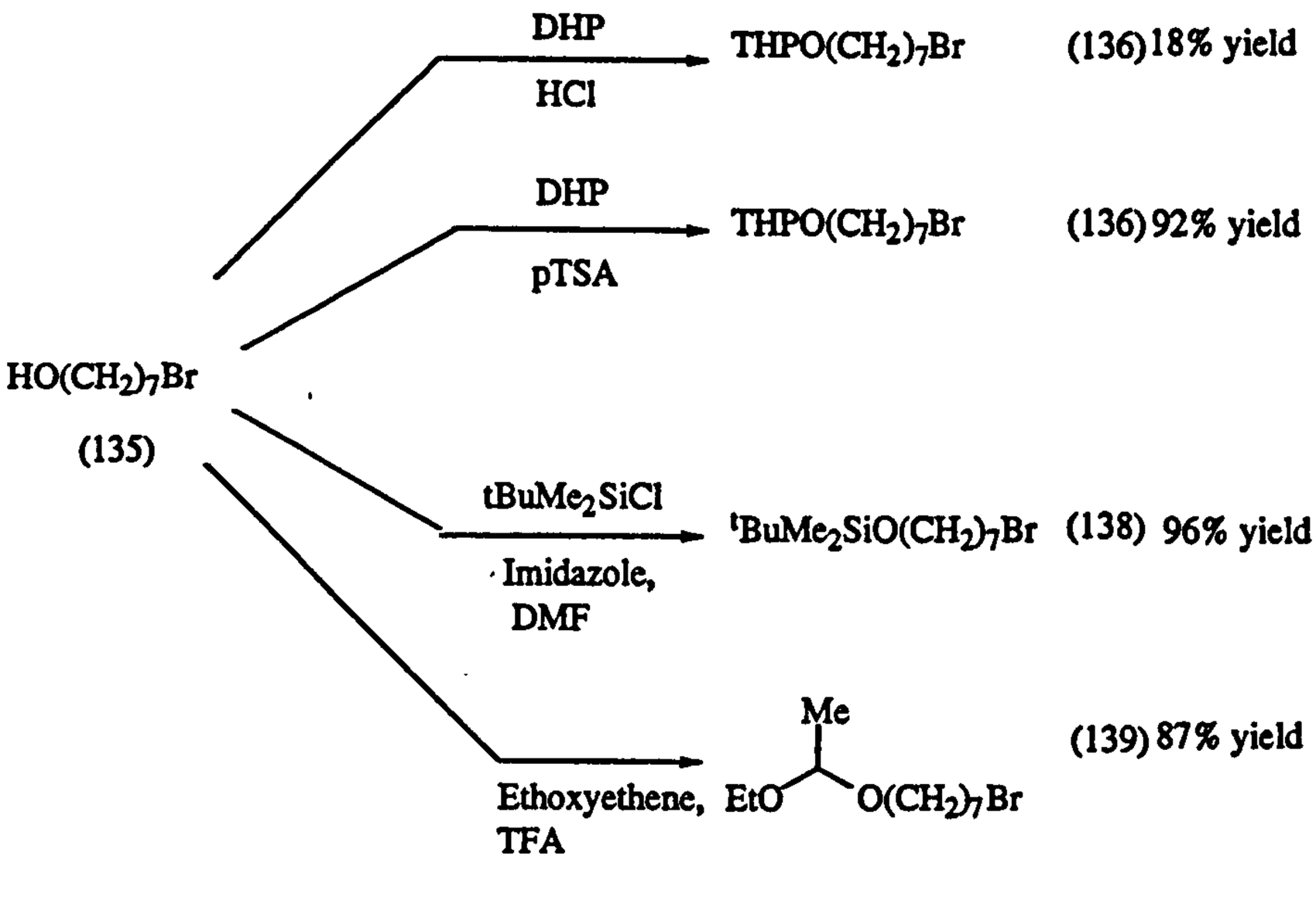
Commercially available 7-bromo-1-heptanol (135) was used as the starting material in the first proposed route for the synthesis of the NAC thioester of [1,2-$^{13}C_2$]-9-hydroxynonanoic acid (Scheme 2.24).



Scheme 2.24: Proposed synthetic route to the NAC thioester (137) of 9-hydroxynonanoic acid.

The first step in the synthesis involved protection of the primary alcohol, and a range of protecting groups was examined (Scheme 2.25).

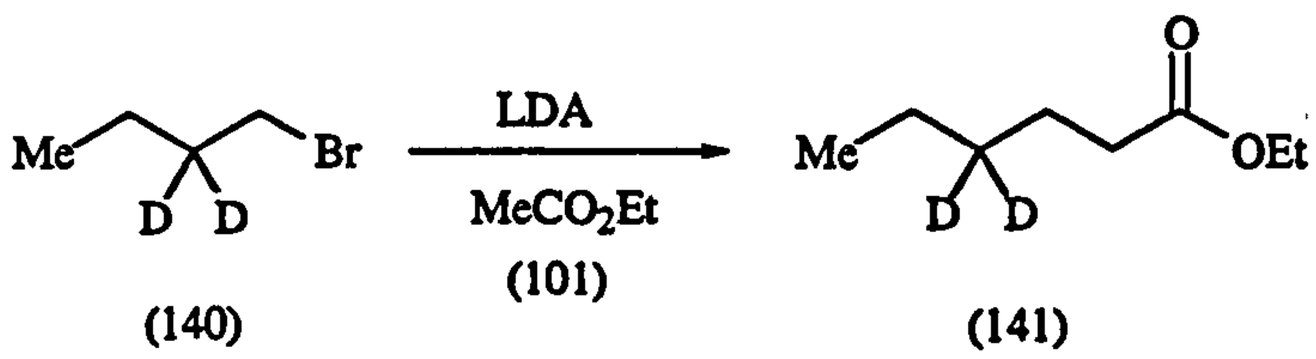


Scheme 2.25: Protection of 7-bromo-1-heptanol.

Protection of the alcohol as the tetrahydropyranyl ether, using the method of Okuno and coworkers,[106] gave (136) in 92% yield, whereas, using the method of Burton

and coworkers,[107] (136) was obtained in only 18% yield.

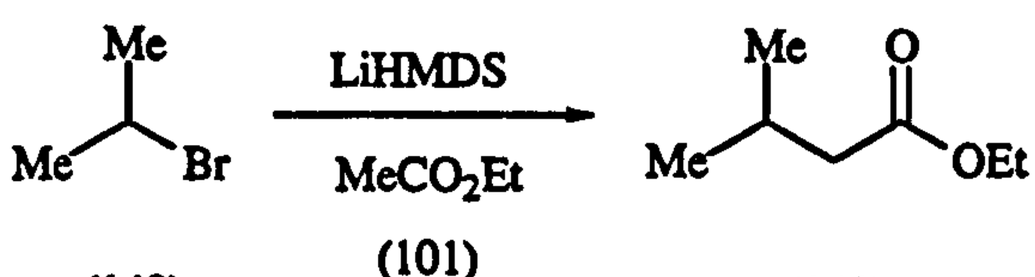
The next step was the alkylation of the protected bromide. McNicholas[108] had already shown that 1-bromobutane (140) could be alkylated in 65% yield, using LDA and ethyl acetate (Scheme 2.26).



(140) (141)

Scheme 2.26: Alkylation of a primary bromide.

Reaction of the bromide with ethyl acetate in the presence of a range of bases, including LDA, LiHMDS, sodium hydride, and sodium ethoxide, gave no reaction. Model reactions, involving short chain bromides were carried out to ensure that the anion of ethyl acetate was being formed. For example, reaction of 2-bromopropane (142) with its anion, generated from ethyl acetate and lithium hexamethyldisilylazide, gave the alkylated product (143) in 72% yield (Scheme 2.27).



(142) (143)

Scheme 2.27: Alkylation of 2-bromopropane.

Alkylation of the protected bromide was then attempted using diethyl malonate in place of ethyl acetate. The best results were obtained, using the procedure reported by Thompson and Reeve,[109] involving a phase-transfer-catalysed alkylation of diethyl malonate. Addition of potassium carbonate and 18-crown-6 to diethyl malonate and the protected bromide gave diethyl-(1'-tetrahydropyran-2-yl)oxynonylmalonate (144) in 98% yield. The ^{1}H nmr spectrum of (144) showed the expected signals at δ 3.31, assigned to 2-H, and δ 1.27 and δ 4.17, due to the diethyl ester.

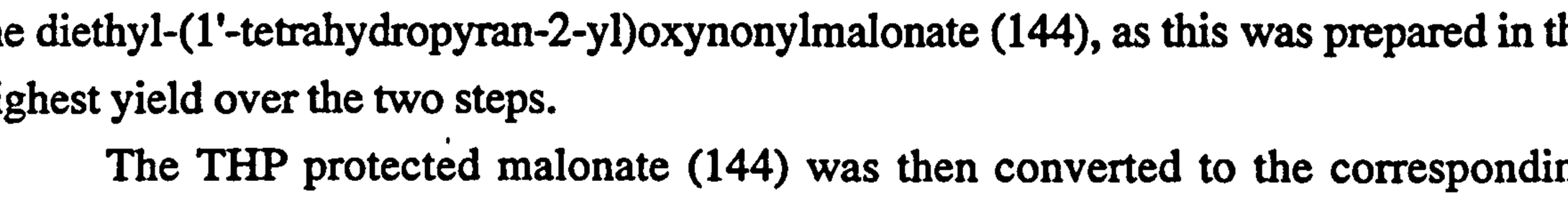
Table 2.2 provides a summary of the various conditions employed for alkylation of the protected 7-bromo-1-heptanol.

Protecting Group	Base	Other reagents	% Yield of Product
THP	LDA	Ethyl acetate	No reaction
THP	LiHMDS	Ethyl acetate	No reaction
THP	Sodium ethoxide	Ethyl acetate	No reaction
THP	Sodium hydride	Ethyl acetate	No reaction
THP	Sodium ethoxide	Diethyl malonate,	20.1
THP	K_2CO_3, 18-Crown-6	Diethyl malonate, CH_3CN	98.0
THP	Sodium hydride	Diethyl malonate, DMF	91.0
TBDMS	LDA	Ethyl acetate	No reaction
TBDMS	LiHMDS	Ethyl acetate	No reaction
TBDMS	K_2CO_3, 18-Crown-6	Diethyl malonate, CH_3CN	65.4
EE	LDA	Ethyl acetate	No reaction
EE	LiHMDS	Ethyl acetate	No reaction
EE	K_2CO_3, 18-Crown-6	Diethyl malonate, CH_3CN	86.9

Table 2.2: Attempted alkylation conditions.

As shown, these alkylations were attempted with three different protecting groups: THP, TBDMS and EE. However, further reactions were carried out only using the diethyl-(1'-tetrahydropyran-2-yl)oxynonylmalonate (144), as this was prepared in the highest yield over the two steps.

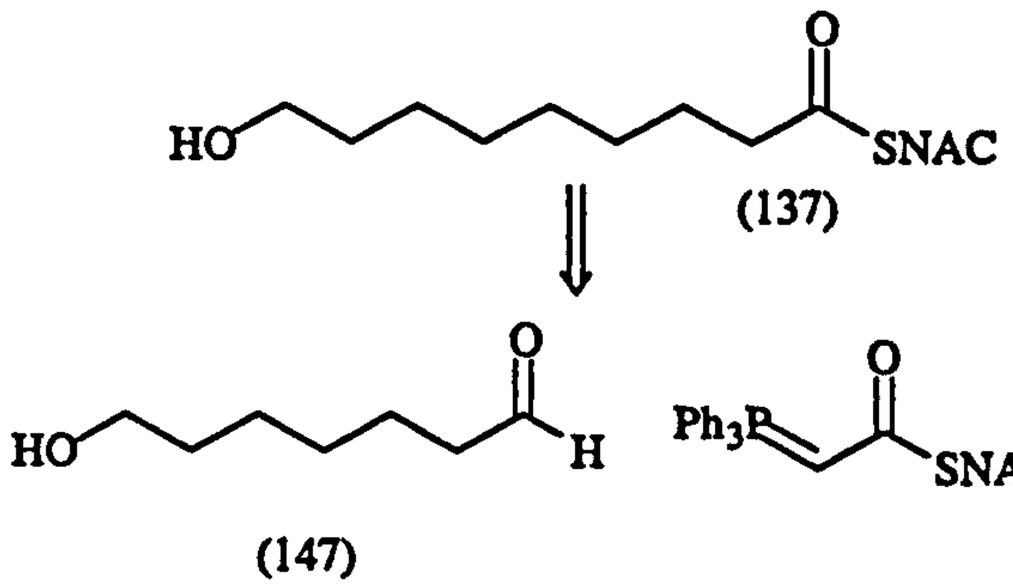
The THP protected malonate (144) was then converted to the corresponding diacid (145) in 89% yield using sodium hydroxide and a phase-transfer catalyst (Scheme 2.28). The ^1H nmr spectrum indicated a triplet at δ 3.41, assigned to 2-H, and loss of a triplet at δ 1.27 and a quartet at δ 4.17, corresponding to loss of the ethyl groups. the mass spectrum gave a molecular ion in accordance with the diacid structure (145).



Scheme 2.28: Synthesis of the protected diacid.

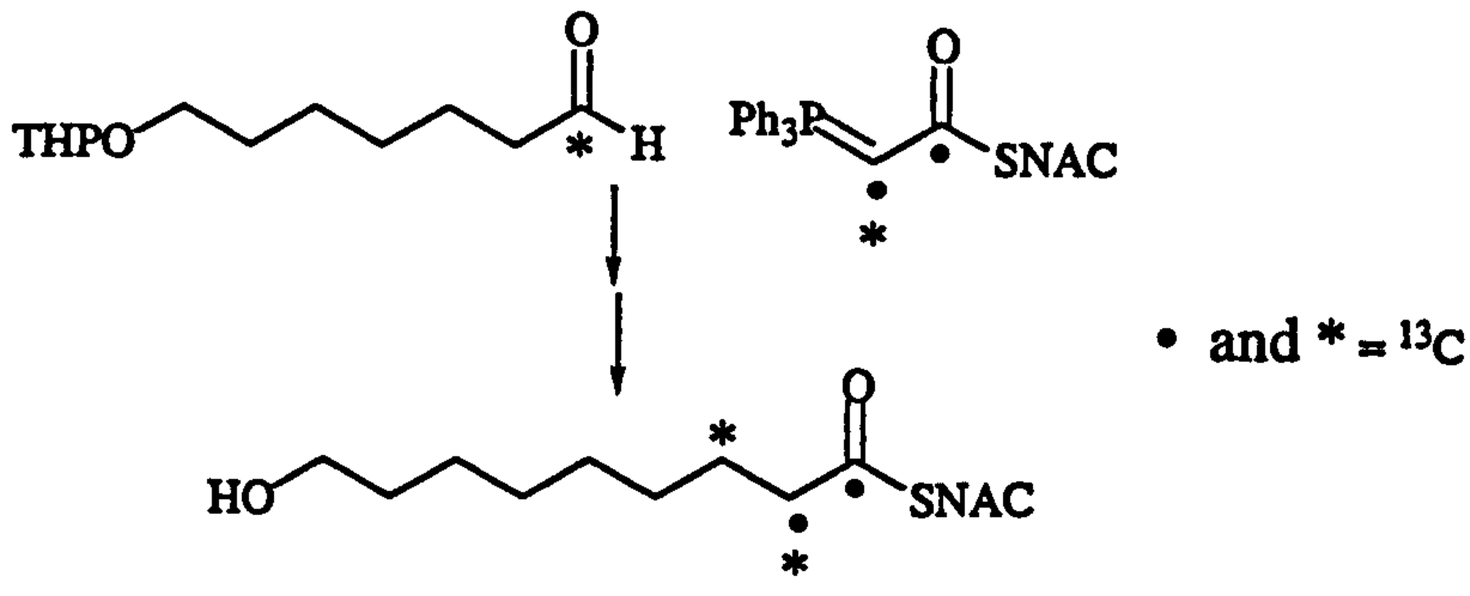
Treatment of the diacid either with acid or in refluxing xylene gave none of the expected decarboxylated product (146). At this stage, this route was abandoned for two reasons. Firstly, in a labelled synthesis of diethyl malonate, a double $^{13}C_2$ labelled malonate is effectively triply labelled, so that on decarboxylation half of the label would be lost. Secondly, this synthesis was only suitable for synthesising the NAC thioester of [1,2-$^{13}C_2$]-9-hydroxynonanoic acid. Meanwhile, another route was being developed, which would lead to both the NAC thioester of [1,2-$^{13}C_2$]-9-hydroxynonanoic acid and the NAC thioester of [2,3-$^{13}C_2$]-9-hydroxynonanoic acid.

Retrosynthetic analysis of the NAC thioester of 9-hydroxynonanoic acid (137) indicated a possible route may be though a Wittig reaction between a C_7 aldehyde (147) and a C_2 phosphorane ylid (Scheme 2.29).



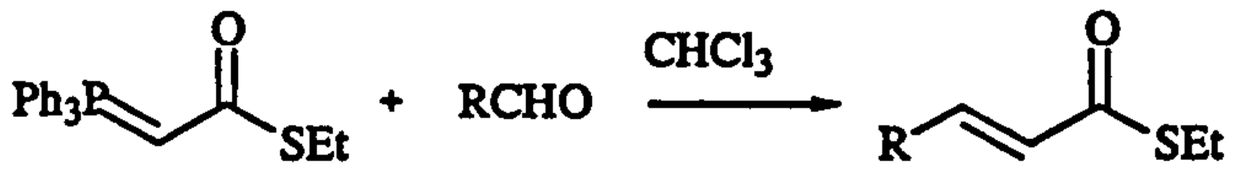
Scheme 2.29: Retrosynthetic analysis of the NAC thioester (137) of 9-hydroxynonanoic acid.

The reaction of aldehydes with Wittig reagents of the structure Ph_3PCHCO_2R constitute an extremely powerful method for two carbon chain extension. This synthesis would hopefully allow the synthesis of the NAC thioester of [1,2-$^{13}C_2$]-9-hydroxynonanoic acid by coupling unlabelled aldehyde with the NAC thioester of [1,2-$^{13}C_2$]triphenylphosphorane acetate. The synthesis of the NAC thioester of [2,3-$^{13}C_2$]-9-hydroxynonanoic acid would be achieved by coupling the corresponding [1-^{13}C] C_7 aldehyde with the NAC thioester of [2-^{13}C]triphenylphosphorane acetate (Scheme 2.30).



Scheme 2.30: Proposed syntheses of the NAC thioesters of [1,2-$^{13}C_2$] and [2,3-$^{13}C_2$]-9-hydroxynonanoic acid.

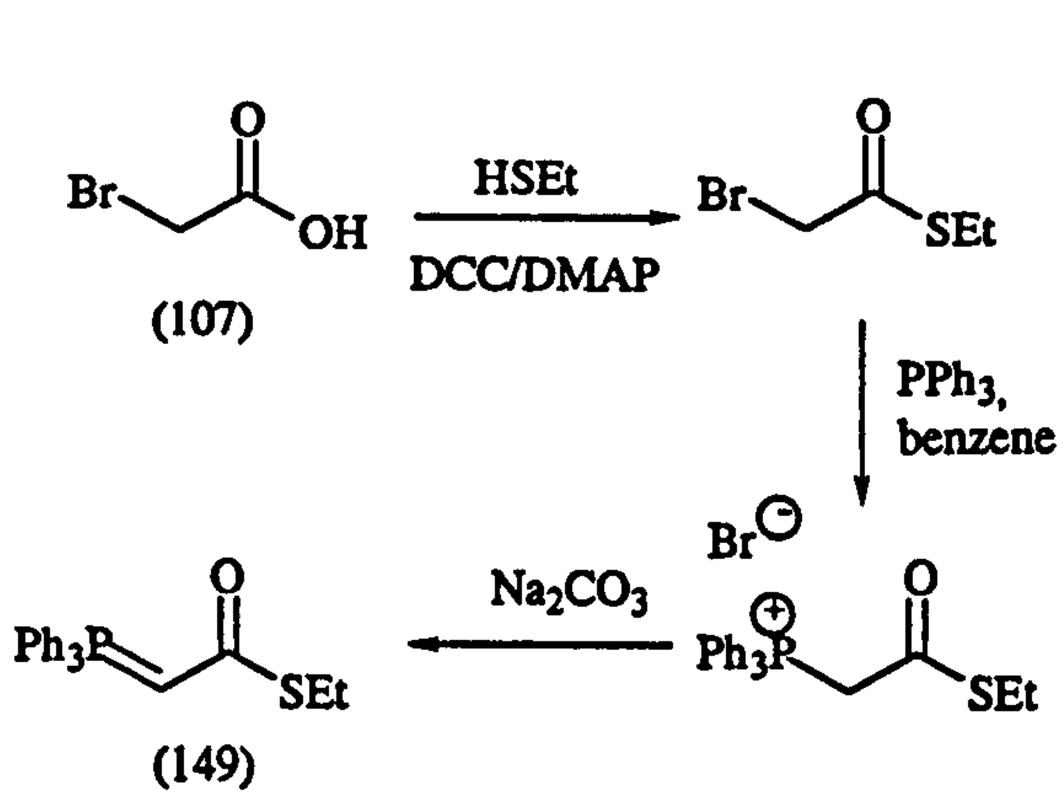
Keck and coworkers[110] reported the preparation and use of the ethane thiol phosphorane (148), with aldehydes (149) to give the unsaturated thioesters (150) (Scheme 2.31).



(148) (149) (150)

Scheme 2.31: Generalised Wittig reaction of the ethane thiol phosphorane.

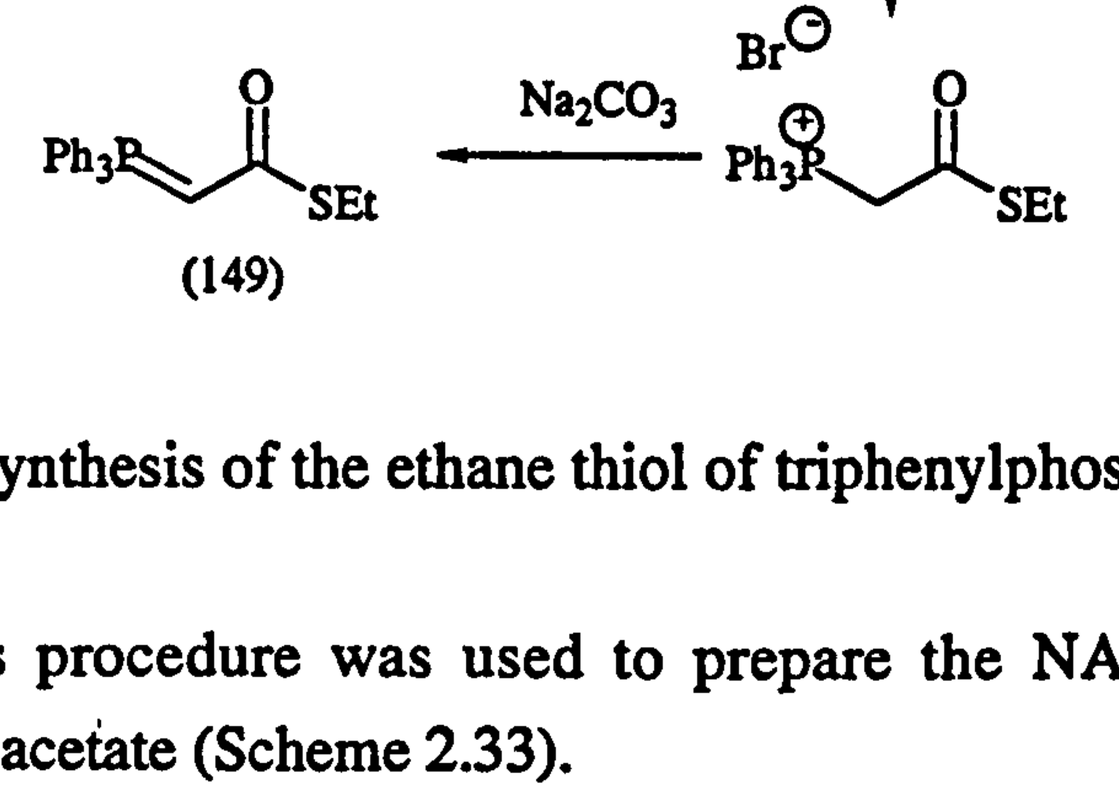
The ethane thiol phosphorane was prepared from bromoacetic acid, as shown in Scheme 2.32. Reaction of bromoacetic acid (107) and ethanethiol, under DCC/DMAP mediated coupling conditions gave the ethane thiol of bromoacetic acid.[83] Subsequent reaction with triphenylphosphine, followed by the addition of 10% sodium carbonate solution gave the required phosphorane (149).



(107) (149)

Scheme 2.32: Synthesis of the ethane thiol of triphenylphosphorane acetate.

An analogous procedure was used to prepare the NAC thioester (151) of triphenylphosphorane acetate (Scheme 2.33).



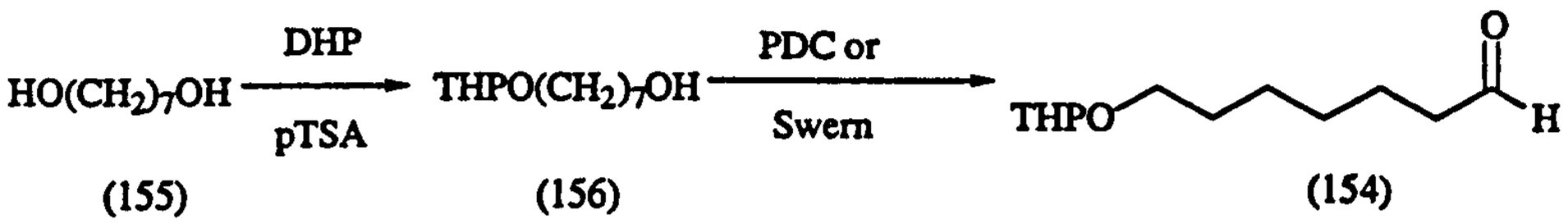
(107) (152) (153) (151)

Scheme 2.33: Synthesis of the NAC thioester of triphenylphosphorane acetate.

Bromoacetic acid (107) was coupled with N-acetylcysteamine in the presence of DCC/DMAP in 85% yield.[83] Reaction of the resultant thioester (152) with triphenylphosphine in toluene produced the NAC thioester of (triphenylphosphonium acetate) bromide (153) in 72% yield. Formation of the required ylid (151) was achieved by titration of (153) with sodium hydroxide.

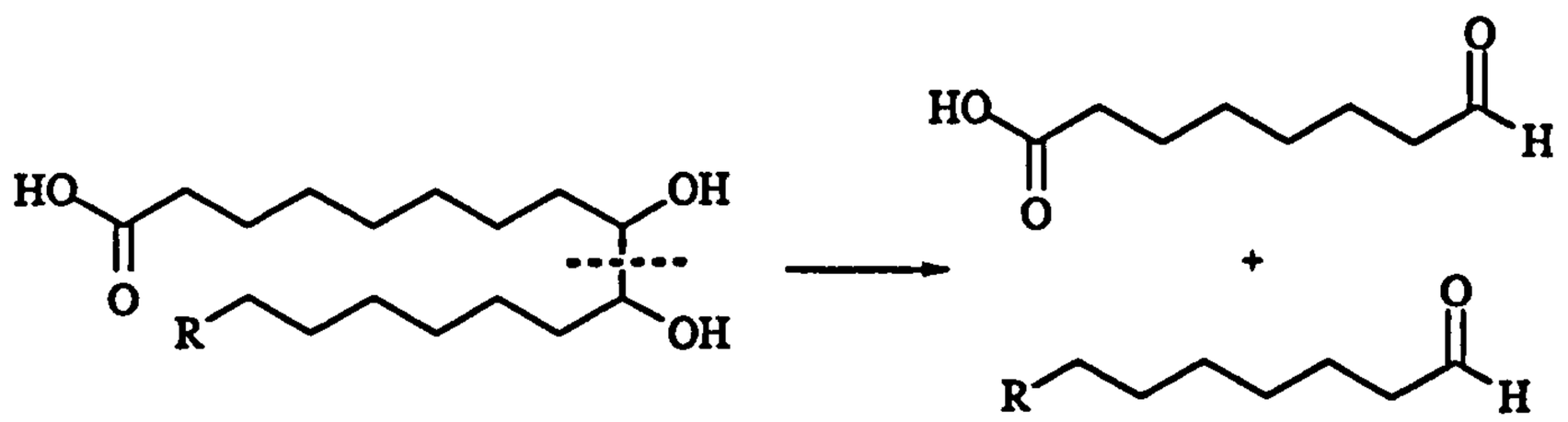
For the second half of the coupling, 7-hydroxyheptanal (147) was required. It was initially thought to protect the hydroxy group as its THP ether. Hence, the target became 7-(1'-tetrahydropyran-2-yl)oxyheptanal (154). Several routes were developed to allow the synthesis of both unlabelled and [1-^{13}C] versions of the protected aldehyde.

7-(1'-Tetrahydropyran-2-yl)oxyheptanal was prepared from readily available 1,7-heptanediol (155). Mono-protection of (155), using dihydropyran and pTSA, as its THP ether was achieved in 39% yield. Oxidation of (156) with PDC or under Swern conditions gave 7-(1'-tetrahydropyran-2-yl)oxyheptanal in 84% and 73% yield respectively (Scheme 2.34).[106,111] Despite the low yield of mono-protected alcohol, the scheme was deemed useful as a cheap, reliable, and quick route to large quantities of the protected aldehyde.



Scheme 2.34: Synthesis of 7-(1'-tetrahydropyran-2-yl)oxyheptanal.

Another route for the preparation of (154) was developed, using the commercially available aleuritic acid as the starting material.[107] Cleavage of aleuritic acid (157) with periodate gave 7-hydroxyheptanal (147) in 83% yield. Protection as its THP ether was achieved in 78% yield, using dihydropyran and pTSA. Comparison of the ^{1}H nmr spectrum with the aldehyde formed via PDC oxidation confirmed successful oxidative cleavage of aleuritic acid (Scheme 2.35).

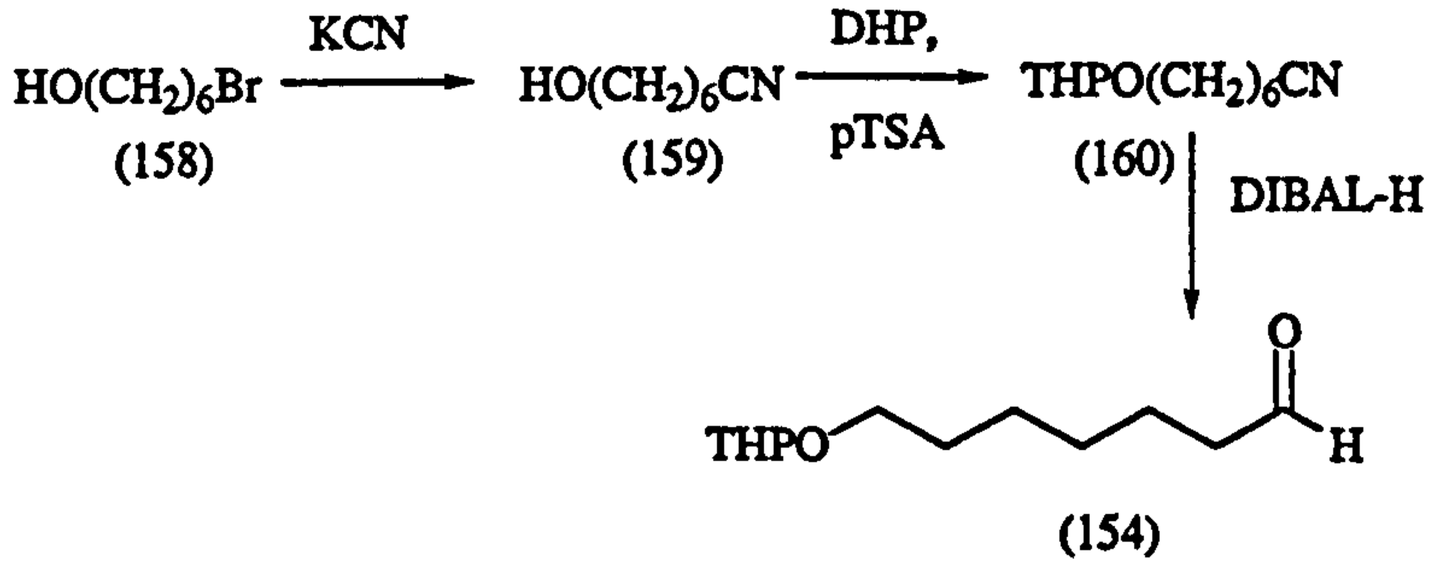


Scheme 2.35: Periodate cleavage of aleuritic acid.

It should be noted that these synthetic routes only allow the synthesis of

unlabelled 7-(1'-tetrahydropyran-2-yl)oxyheptanal, and so can only be used in conjunction with the NAC thioester of [1,2-$^{13}C_2$]triphenylphosphorane acetate in the synthesis of the NAC thioester of [1,2-$^{13}C_2$]-9-hydroxynonanoic acid.

Another route was therefore developed for the preparation of [1-^{13}C]-7-(1'-tetrahydropyran-2-yl)oxyheptanal, to be used in the preparation of the NAC thioester of [2,3-$^{13}C_2$]-9-hydroxynonanoic acid (Scheme 2.36). The source of the isotopic label was to be potassium [1-^{13}C]cyanide, but first the route was optimised using unlabelled material.



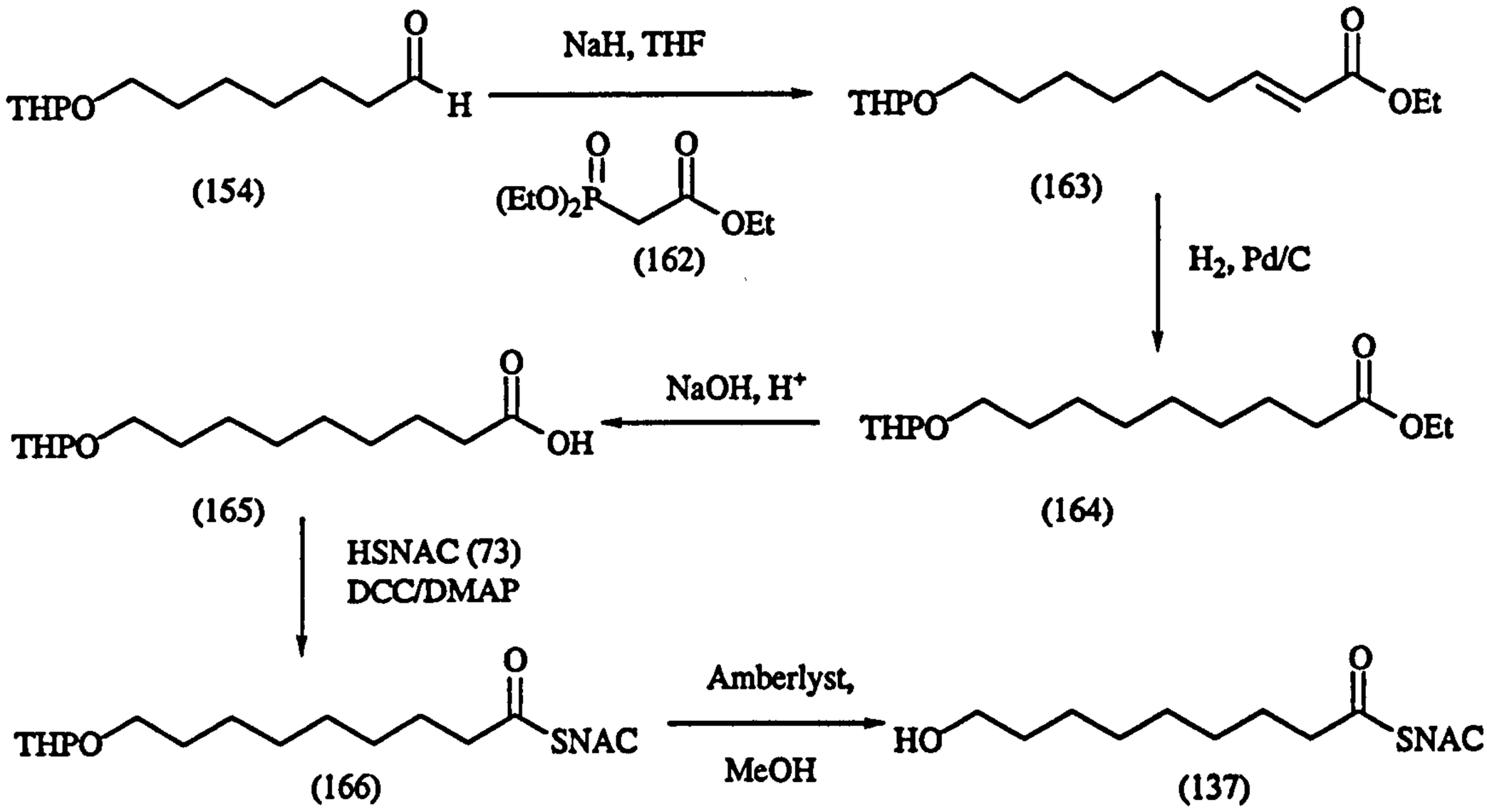
Scheme 2.36: Synthesis of 7-(1'-tetrahydropyran-2-yl)oxyheptanal, via DIBAL reduction of 7-(1'-tetrahydropyran-2-yl)oxyheptanitrile (160).

Treatment of 6-bromo-1-hexanol (158) with sodium cyanide gave 7-hydroxyheptanitrile (159) in 69% yield. An upfield shift of the triplet (*J* 7Hz) from δ 3.4 to δ 2.4 assigned to 6-methylene in (159), indicating that the nitrile had been formed. This was confirmed by ^{13}C nmr, in which the signal at δ 119 was assigned to the nitrile carbon. Protection of the primary alcohol as the THP ether gave 7-(1'-tetrahydropyran-2-yl)oxyheptanitrile (160) in 60% yield. Addition of 1.5 equivalents of DIBAL-H solution to (160) led to the successful formation of 7-(1'-tetrahydropyran-2-yl)oxyheptanal (154) in 73% yield.

The Wittig coupling between the protected aldehyde (154) and the phosphorane (151) was carried out in refluxing chloroform, giving the required α,β unsaturated thioester (161) in 45% yield. The ^{1}H nmr spectrum of (161) confirmed that the *trans* double bond had been formed, showing the signals at δ 5.80 and 6.95 were coupled to each other with a coupling constant of 15.4 Hz. A small allylic coupling of 1.5 Hz was also observed at δ 5.80, resulting in a doublet of triplets.

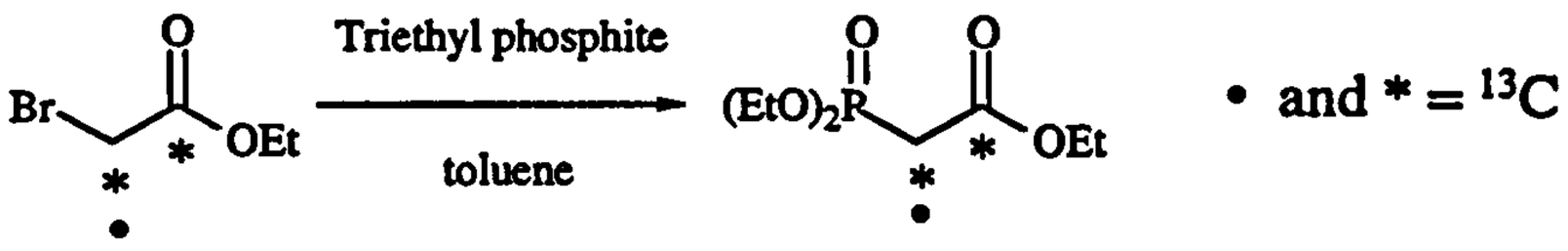
Subsequent hydrogenation, using a palladium/carbon catalyst was unsuccessful, with the return of starting material. This is possibly due to poisoning of the catalyst by the presence of the sulphur atom.

Hence an alternative approach for the synthesis of the NAC thioester of 9-hydroxynonanoic acid was required, and use of a more divergent route was investigated (Scheme 2.37).



Scheme 2.37: Proposed coupling, and subsequent modifications to synthesise the NAC thioester of 9-hydroxynonanoic acid.

A Horner-Wadsworth-Emmons reaction with ethyl diethylphosphonacetate (162) was to be used for the 2 carbon chain homologation of the aldehyde (154). Reaction of ethyl bromoacetate with triethyl phosphate, in toluene, gave ethyl phosphonoacetate in 79% yield (Scheme 2.38). By use of either ethyl [1,2-$^{13}C_2$] or [2-^{13}C]bromoacetate, the required isotopically labelled phosphonate may be prepared.

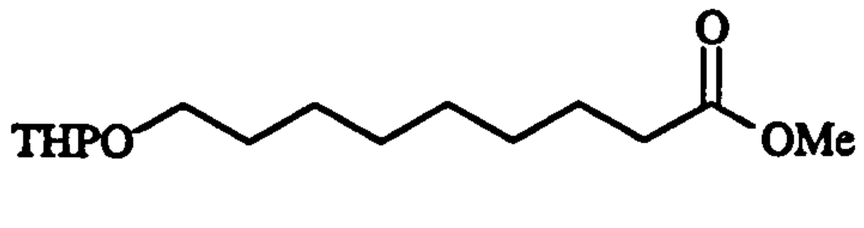


Scheme 2.38: Synthesis of ethyl phosphonoacetate, enabling incorporation of a single or double ^{13}C label.

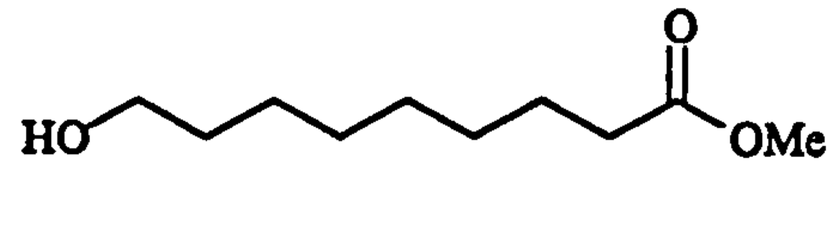
Chain extension of the protected aldehyde (154) with ethyl phosphonoacetate was achieved using 1.5 equivalents of sodium hydride giving ethyl 9-(1'-tetrahydropyran-2-yl)oxynon-(2*E*)-enoate (163) in 62% yield. Hydrogenation of (163), using a palladium/carbon catalyst in methanol, produced the saturated ethyl ester (164) in 71% yield. As expected, the ^{1}H nmr spectrum of (164) showed no signals at δ 5.80 and 7.0, confirming that the saturated ester (164) had been formed. Base hydrolysis of (164), using a phase-transfer catalyst, gave the THP protected 9-hydroxynonanoic acid (165) in 78% yield.

At this point, DCC/DMAP mediated coupling of the THP protected 9-hydroxynonanoic acid with N-acetylcysteamine produced the THP protected NAC thioester (166) of 9-hydroxynonanoic acid in a crude yield of 93%. Subsequent

deprotection of the THP protecting group was attempted using the amberlyst-15 and methanol, as already described. However, instead of deprotection of the THP group, 9-(1'-tetrahydropyran-2-yl)oxynonanoate (167) was isolated in 77% yield, presumably due to preferential attack of the methanol on the carboxyl carbon, thus displacing the thioester group. Deprotection of the THP group gave methyl 9-hydroxynonanoate (168) in 65% yield. Subsequent base hydrolysis gave 9-hydroxynonanoic acid (69) in 61% yield.



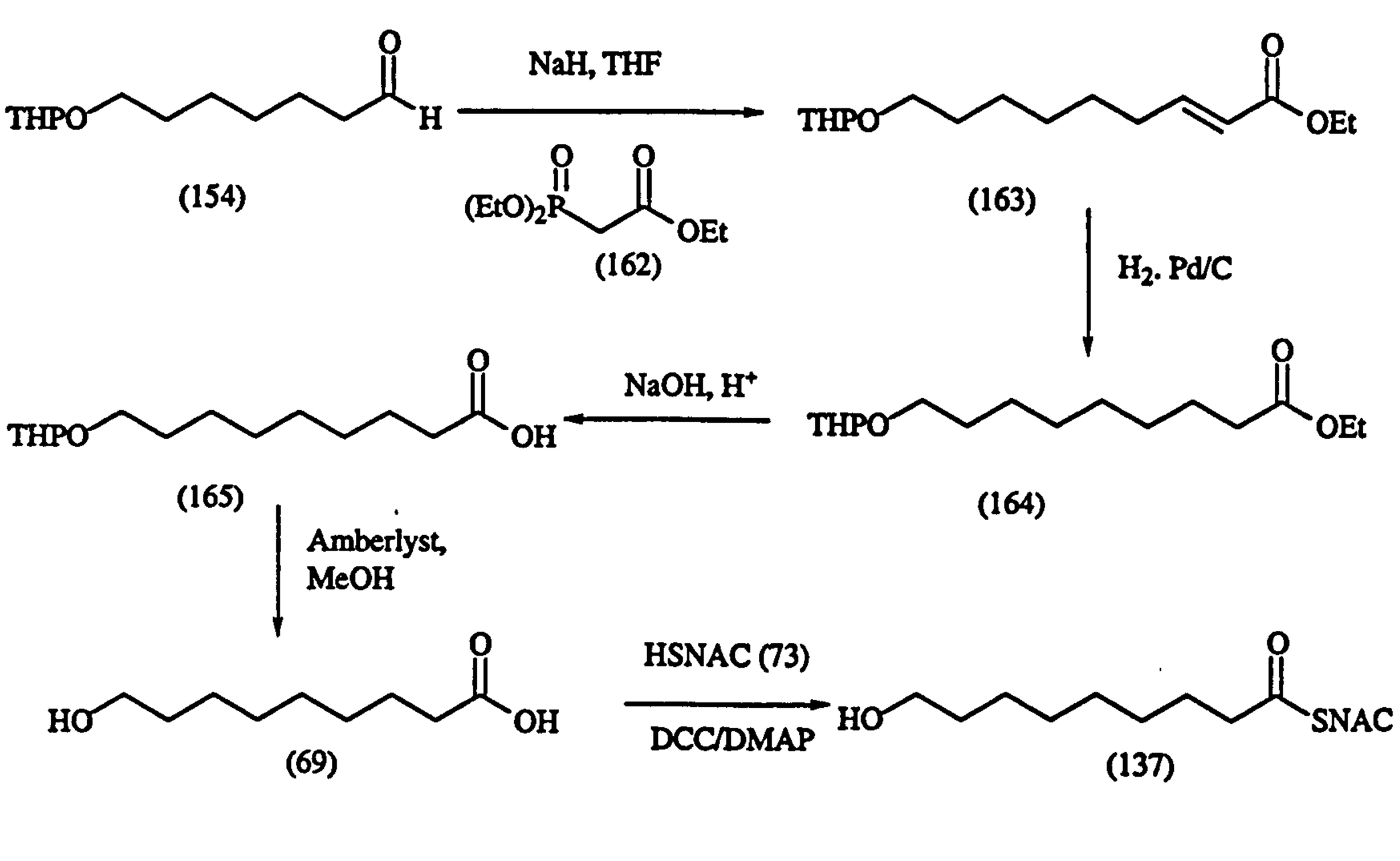
(167)



(168)

At this point, it was decided to return to the THP protected 9-hydroxynonanoic acid (165), and attempt a similar deprotection. Removal of the THP ether was achieved by treatment of (165) with amberlyst-15 in methanol to give 9-hydroxynonanoic acid (69) in 49% yield. The final step was the DCC/DMAP mediated coupling of N-acetylcysteamine and 9-hydroxynonanoic acid, which gave the NAC thioester (137) of 9-hydroxynoanoic acid in 79% yield.

This route (Scheme 2.39) enables the synthesis of both the NAC thioesters of [1,2-$^{13}C_2$] and [2,3-$^{13}C_2$]-9-hydroxynonanoic acid.

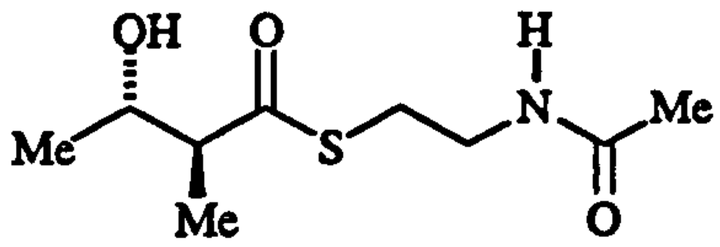


Scheme 2.39: Finalised synthetic route to the NAC thioester of 9-hydroxynonanoic acid.

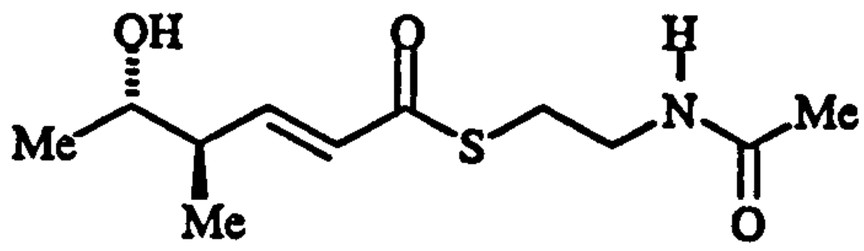
2.4 Synthesis of isotopically labelled intermediates required to investigate the biosynthesis of monic acid

As discussed in chapter 1, previous feeding studies have suggested that monic acid is polyketide in origin, and a processive mode of assembly has been postulated (Scheme 1.27). To summarise this assembly pathway, an acetate and malonate condensation produces enzyme bound acetoacetate (20). Subsequent methylation and reduction gives the enzyme bound diketide, (2*S*,3*S*)-3-hydroxy-2-methylbutanoate (70), which on further condensation, reduction, and dehydration leads to the (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enoate enzyme bound moiety (71). Further condensations with the appropriate number of reductive modifications lead to the putative heptaketide (73), prior to PKS translation to the enzyme bound monic acid (65).

In order to determine the validity of this scheme, isotopically labelled diketide, and triketide were chosen as targets for synthesis, as their NAC thioesters (169) and (170), and subsequent incorporation studies.



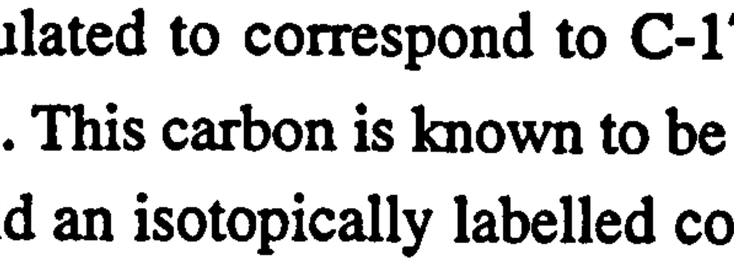
(169)



(170)

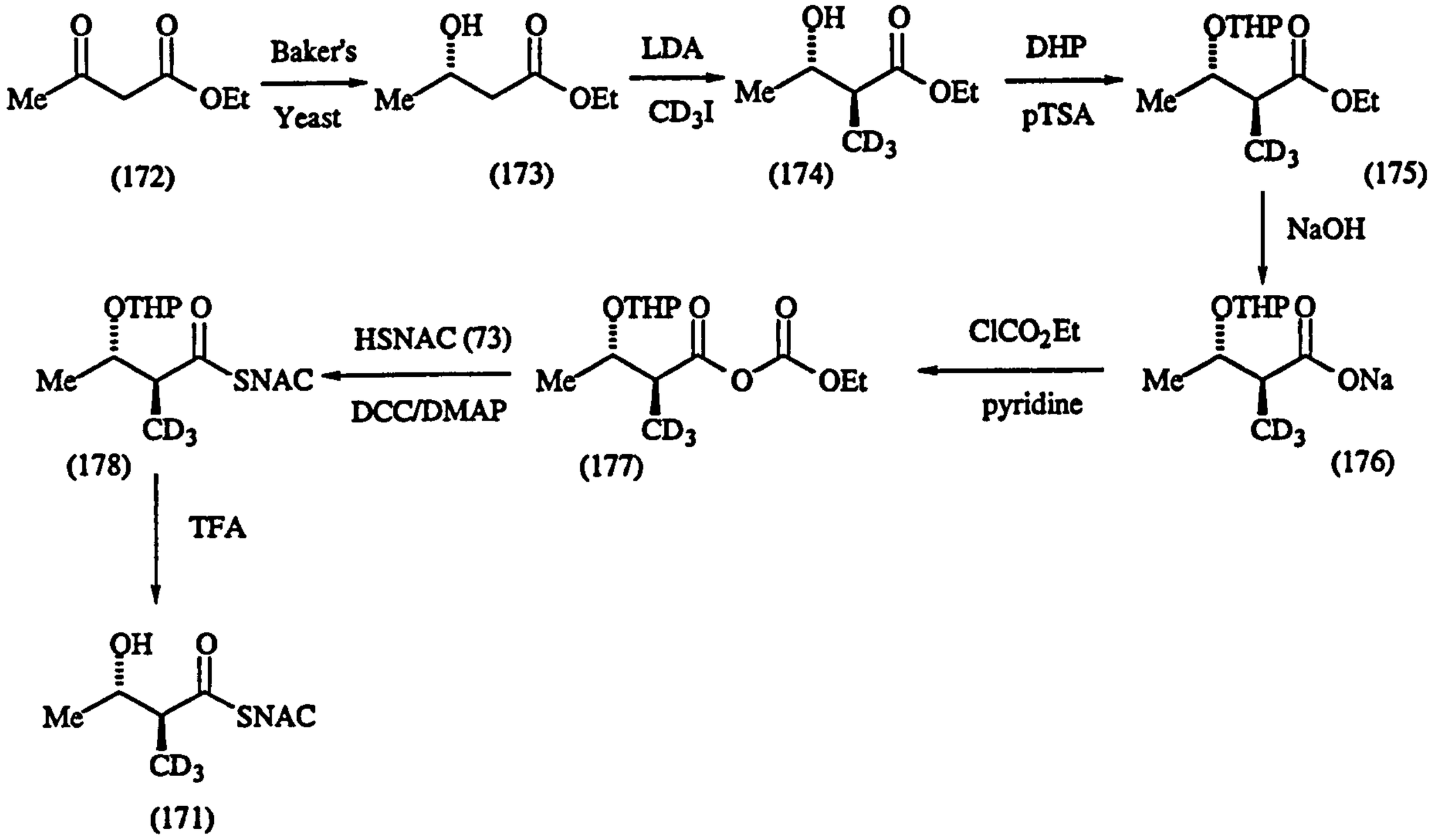
Once again, the position of the label was important, to ensure the experiments could distinguish between catabolism, and intact incorporation.

The branching methyl group of the NAC thioester (169) of (2*S*,3*S*)-3-hydroxy-2-methylbutanoic acid is postulated to correspond to C-17 of monic acid (50), by the processive mode of assembly. This carbon is known to be biosynthetically derived from methionine. Therefore, should an isotopically labelled compound undergo β-oxidation after administration to *Pseudomonas fluorescens*, the label would not be expected to appear at C-17 in the isolated pseudomonic acid, as β-oxidation leads only to acetate. Therefore, if a label is introduced into the branching methyl group, detection of a label at C-17 would imply intact incorporation of this compound. The initial target molecule was therefore the NAC thioester (171) of [2-$^{2}H_3$]-(2*S*,3*S*)-3-hydroxy-2-methylbutanoic acid.



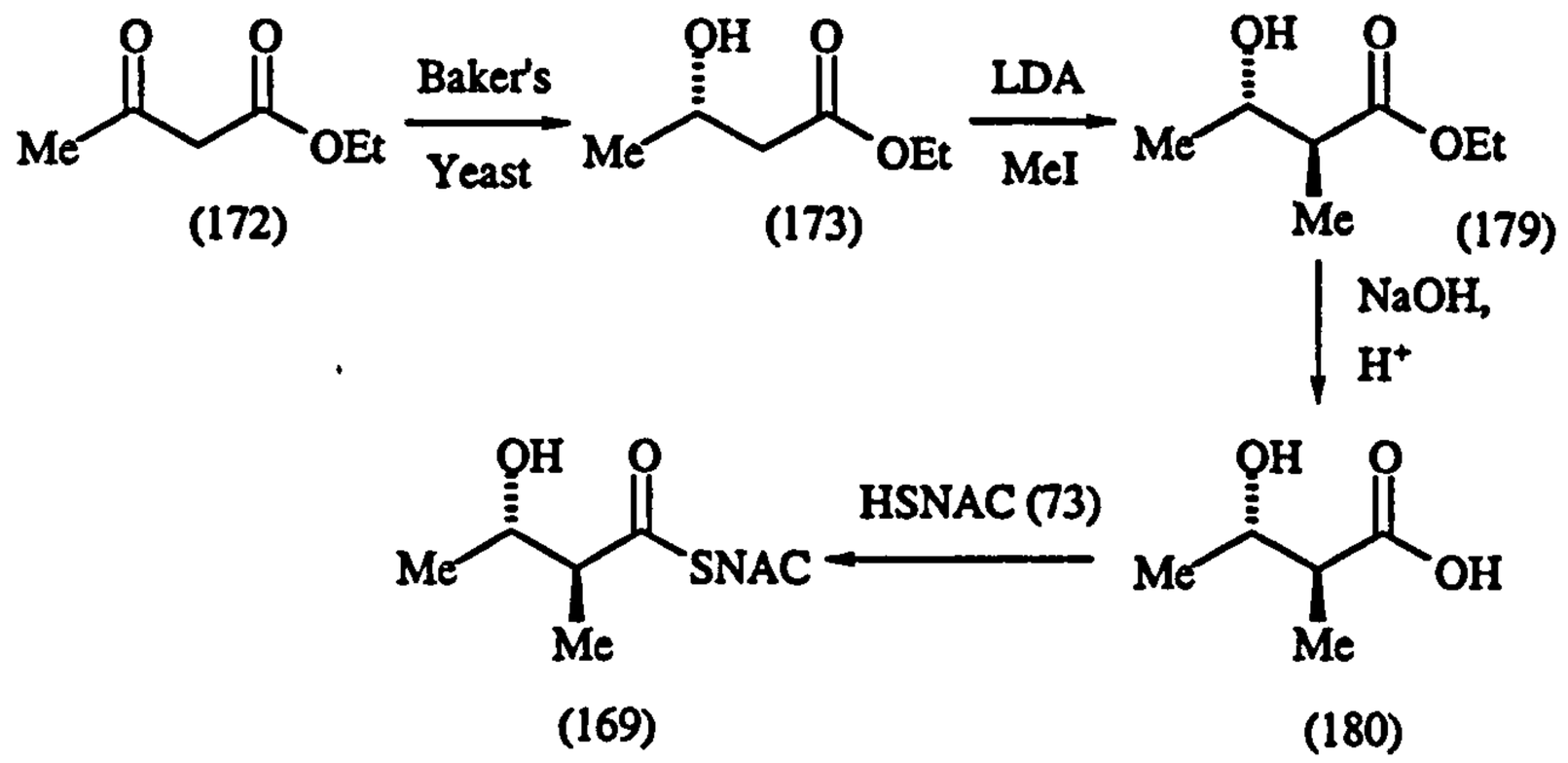
(171)

The compound has been previously prepared by Martin using [$^{2}H_3$] methyl iodide as the source of isotopic label (Scheme 2.40).[71]



Scheme 2.40: Martin's synthesis of the NAC thioester of (2*S*,3*S*)-3-hydroxy-2-methylbutanoic acid

Work in our laboratories, on related systems, has made direct use of DCC/DMAP mediated coupling of a free acid with N-acetylcysteamine.[83] Using this methodology, a more direct route to the required thioester was examined, via the free acid (180) rather than the mixed anhydride (177) (Scheme 2.41).



Scheme 2.41: Synthesis of the NAC thioester of (2*S*,3*S*)-3-hydroxy-2-methylbutanoic acid.

The required stereochemistry was introduced in the first step, by means of a Baker's yeast reduction of the readily available ethyl acetoacetate (172) giving ethyl (3*S*)-3-hydroxybutanoate (173) in 53% yield.[112] This method allowed large quantities of this compound to be prepared. However, the enantiomeric excess was estimated to be 70%,

based on the α_D reading obtained, and comparing it with a known literature value.

Methylation, using LDA and methyl iodide was carried out producing ethyl (2*S*,3*S*)-3-hydroxy-2-methylbutanoate (179) in 54% yield. As outlined in Fig. 2.6, the presence of the methyl group causes facial bias in the electrophilic attack of the methyl group.[113]

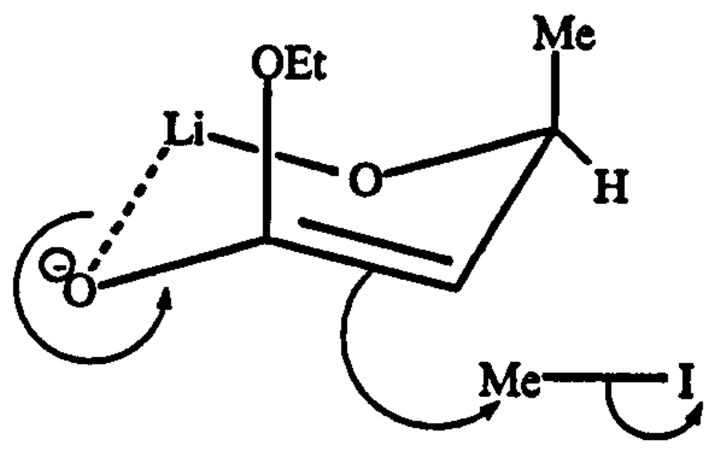
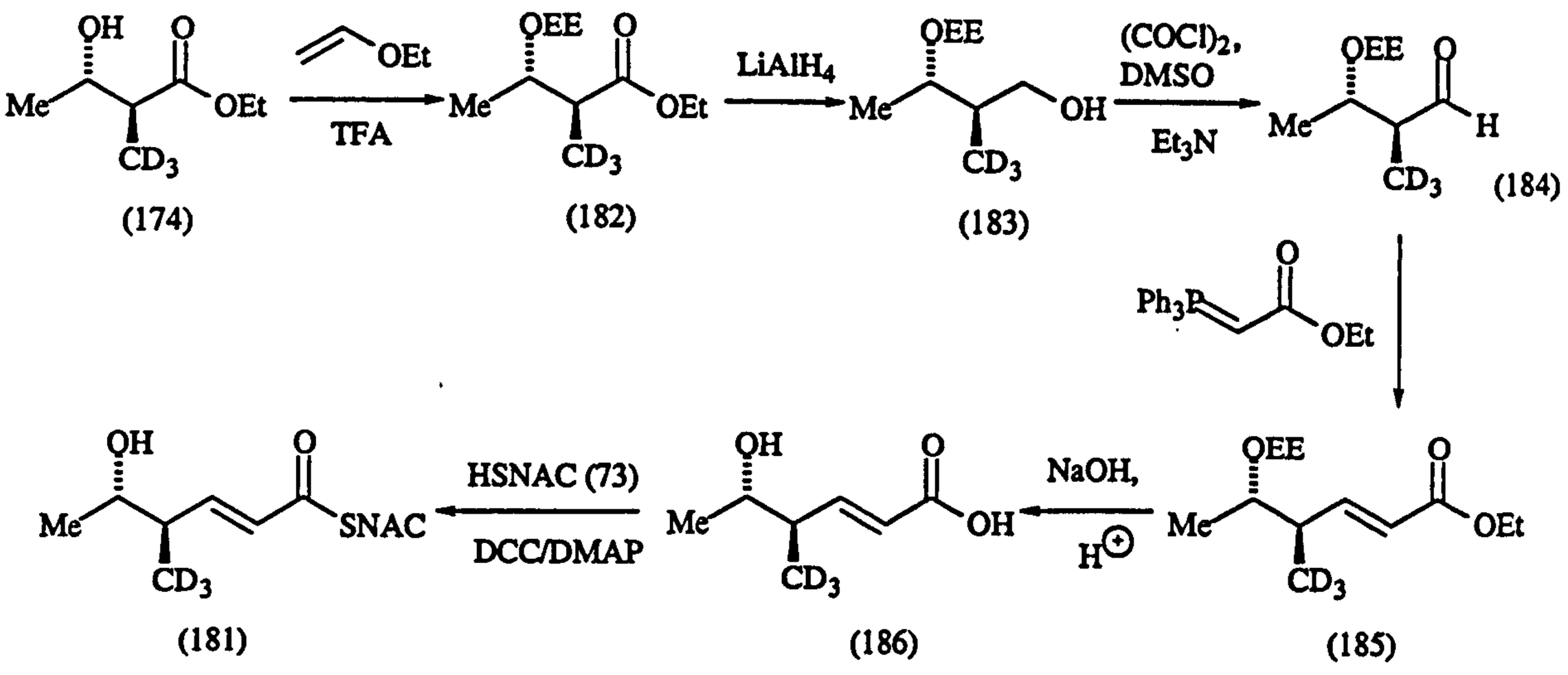


Fig 2.6: Methylation of ethyl (3*S*)-3-hydroxybutanoate, using LDA.

Saponification of ethyl (2*S*,3*S*)-3-hydroxy-2-methylbutanoate (179) gave the corresponding free acid (180) in 92% yield, which was successfully converted to the NAC thioester (169) of (2*S*,3*S*)-3-hydroxy-2-methylbutanoic acid in 25% yield, using DCC/DMAP mediated coupling.[83]

The NAC thioester (181) of [4-$^2H_3$]-(2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enoic acid has been prepared by Sugden,[77] who reported a linear synthesis of this compound (Scheme 2.42).

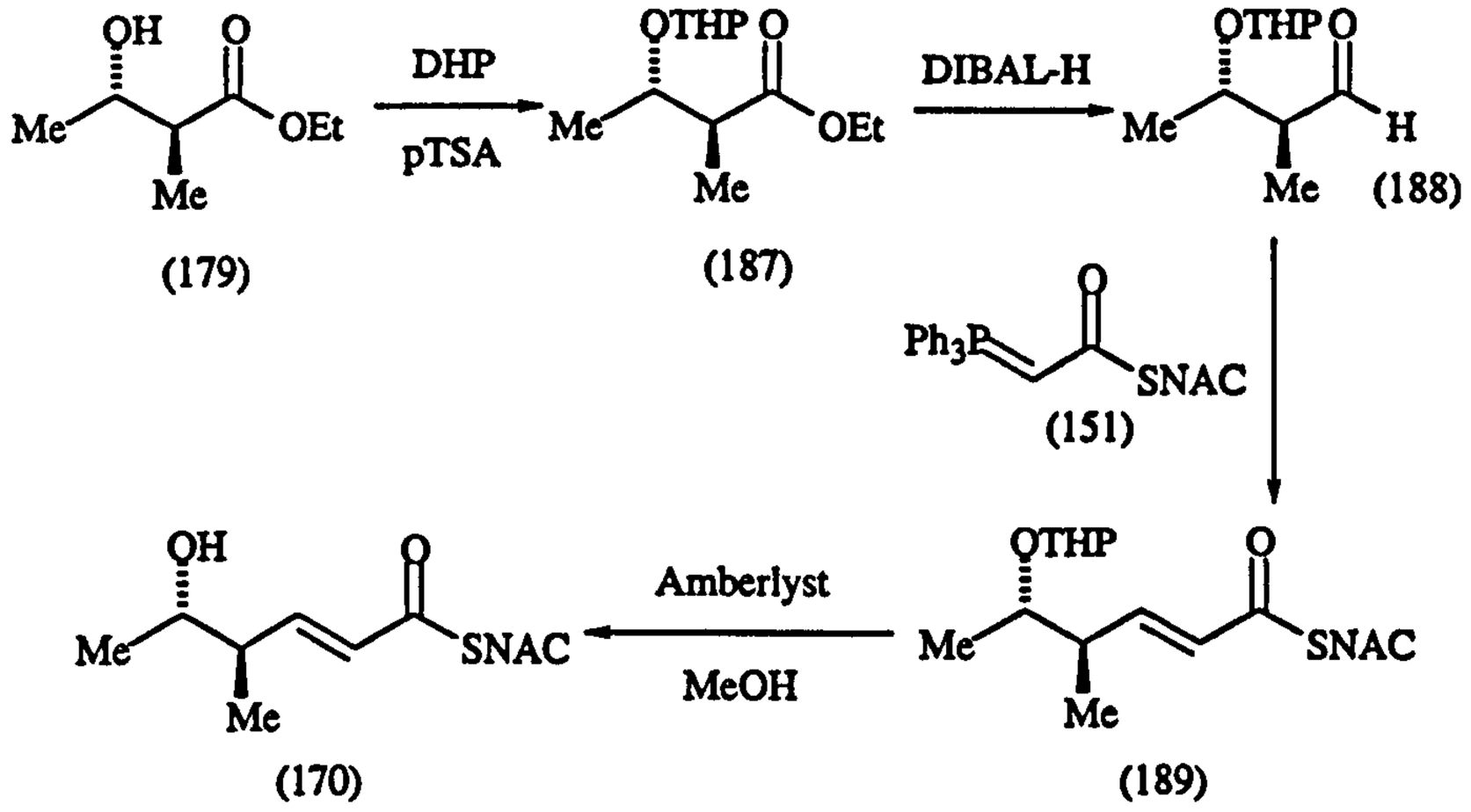


Scheme 2.42: Linear synthesis of the NAC thioester (181) of (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enoic acid.

In order to reduce the number of steps, subsequent to the addition of isotopically labelled material, Sugden[77] prepared the NAC thioester (170) of (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enoic acid, as shown in Scheme 2.43.

Scheme 2.43: Sugden's convergent synthesis of the NAC thioester (170) of (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enoic acid.

A synthetic route of increased convergence, based on further modifying Sugden's synthesis was developed (Scheme 2.44).



Scheme 2.44: Synthesis of the NAC thioester of (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enoic acid.

Ethyl (2*S*, 3*S*)-3-hydroxy-2-methylbutanoate (179) was protected as its THP ether (187), by using dihydropyran and a catalytic amount of pTSA, in 95% yield. Reaction of (187) with 1 equivalent of DIBAL-H gave the aldehyde (188) in 86% yield. The ^{1}H nmr spectrum of (188) showed doublets (*J* 2.4Hz) at δ 9.74 and 9.77, assigned to the aldehydic protons of the two THP diastereomers of (188).

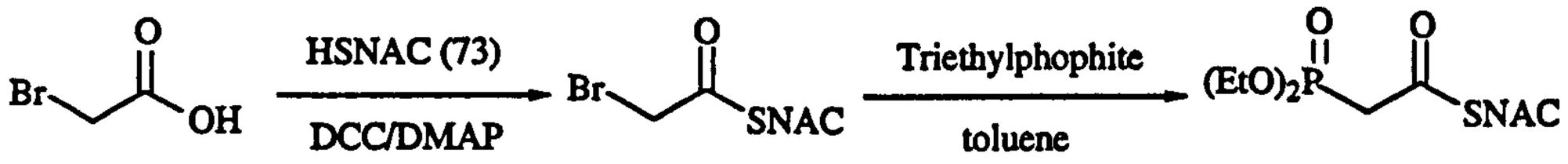
A Wittig reaction[114] was then carried out between the NAC thioester (151) of triphenylphosphorane acetate and the aldehyde in refluxing chloroform. The α,β unsaturated thioester (189) was formed in a crude yield of 92%. The ^{1}H nmr spectrum showed four sets of signals at δ 6.13 and 6.15 (each dt, *J* 15.4 Hz, 0.5 Hz), and δ 6.92

and 6.97 (each dt, *J* 15.4 Hz, 5.5 Hz), indicating the presence of the *trans* unsaturated bond.

Successful deprotection of the THP protecting group was achieved using amberlyst-15 and methanol in 27% yield, giving the required thioester (170). The ^{1}H nmr spectrum of the deprotected thioester was simplified, compared with the THP protected thioester (188). Since it no longer is a mixture of diastereomers. For example, the olefin protons now were apparent simply as two sets of doublets of triplets at δ 6.15 (*J* 15.4 Hz, 1 Hz) and δ 6.92 (*J* 15.4 Hz, 7.8 Hz)

More recently, it has also been found in our laboratories that TBDMS and EE protecting groups may be used in conjunction with these and related systems. The analysis of the ^{1}H nmr of these compounds is much easier than the corresponding ^{1}H nmr spectra obtained when using the THP protecting group.

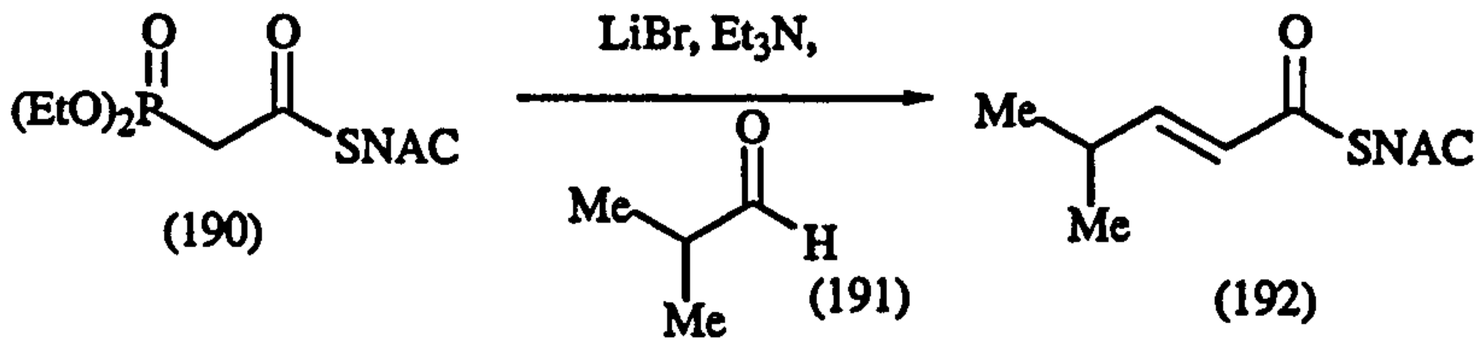
A problem inherent in the synthesis of (170), shown in Scheme 2.44, is that the triphenylphosphine oxide formed as a by product in the Wittig reaction has proved difficult to remove. Staunton and coworkers[30] have reported similar problems. They overcame the problem using an approach based on the Horner-Wadsworth-Emmons reaction.[115] Initially, bromoacetic acid (107) was coupled with N-acetylcysteamine (73) in 64% yield, and the product was converted, via the Arbusov reaction,[116] to the NAC thioester (190) of diethylphosphonoacetic acid in 69% yield (Scheme 2.45). This route was repeated, and was found to be successful. The NAC thioester of bromoacetic acid (152) was prepared in 85% yield, and conversion to the NAC thioester of diethylphosphonoacetic acid occurred in 79% yield.



(107) (152) (190)

Scheme 2.45: Synthesis of the NAC thioester diethylphosphonoacetic acid.

Staunton and coworkers[30] reported the successful reaction of this thioester with a number of aldehydes, including *iso*-butraldehyde (191) (Scheme 2.46), and so this would be an alternative approach to the Wittig reaction for the 2 carbon chain homologation of our aldehyde (154).

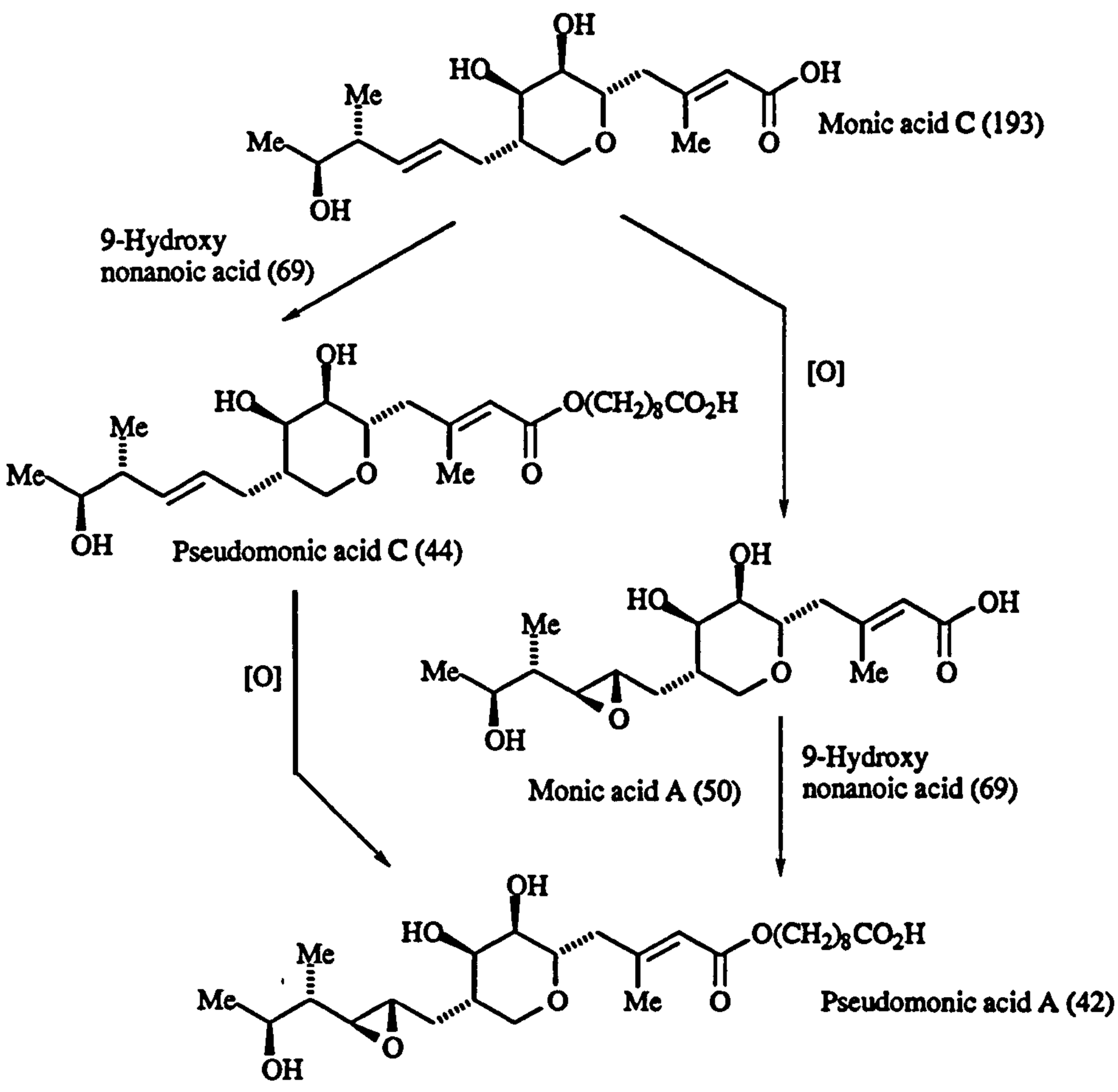


(190) (191) (192)

Scheme 2.46: Synthesis of an α,β unsaturated thioester.

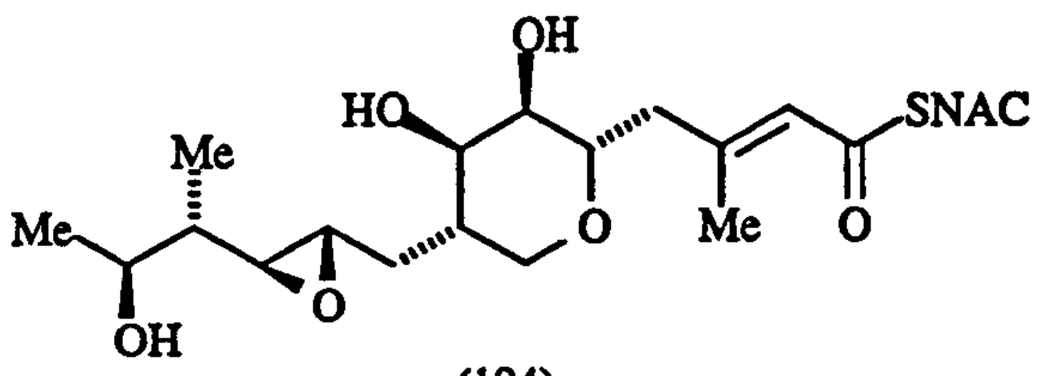
2.5 Synthesis of the monic acid moiety of pseudomonic acid

Pseudomonic acid C (44) is a co-metabolite of pseudomonic acid A (42), and has an identical carbon backbone. The difference in the two structures is that the epoxide moiety at C-10,11 has been replaced by a *trans* double bond. It is more likely that epoxidation of the double bond leads to pseudomonic acid A *in vivo*, as opposed to the deoxygenation of pseudomonic acid A leading to pseudomonic acid C. However, such an epoxidation step may occur prior or subsequent to any esterification, as shown in Scheme 2.47.

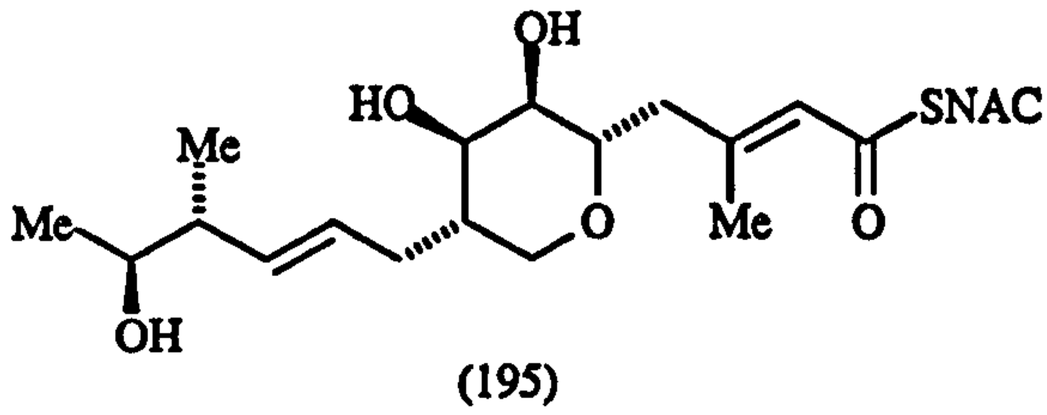


Scheme 2.47: Relative timing of epoxidation and esterification steps on the biosynthetic pathway to pseudomonic acid.

Using the methodology developed in this work, the NAC thioester of 9-hydroxynonanoic acid can now be synthesised in doubly ^{13}C-labelled form. Synthetic routes to the NAC thioester (194) of monic acid A and the NAC thioester (195) of monic acid C were therefore required, in order to fully investigate the timing of these biosynthetic events.

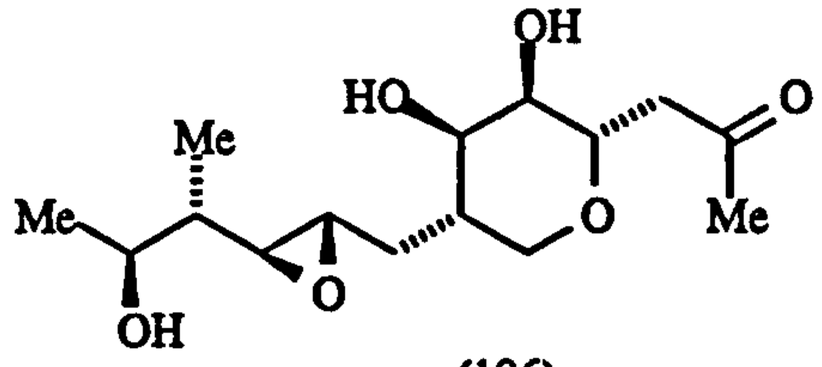


(194)



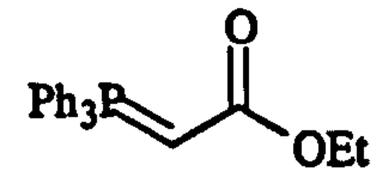
(195)

Methyl ketone (196) has proved to be a valuable intermediate for the preparation of pseudomonic acid and its analogues.[117,118]



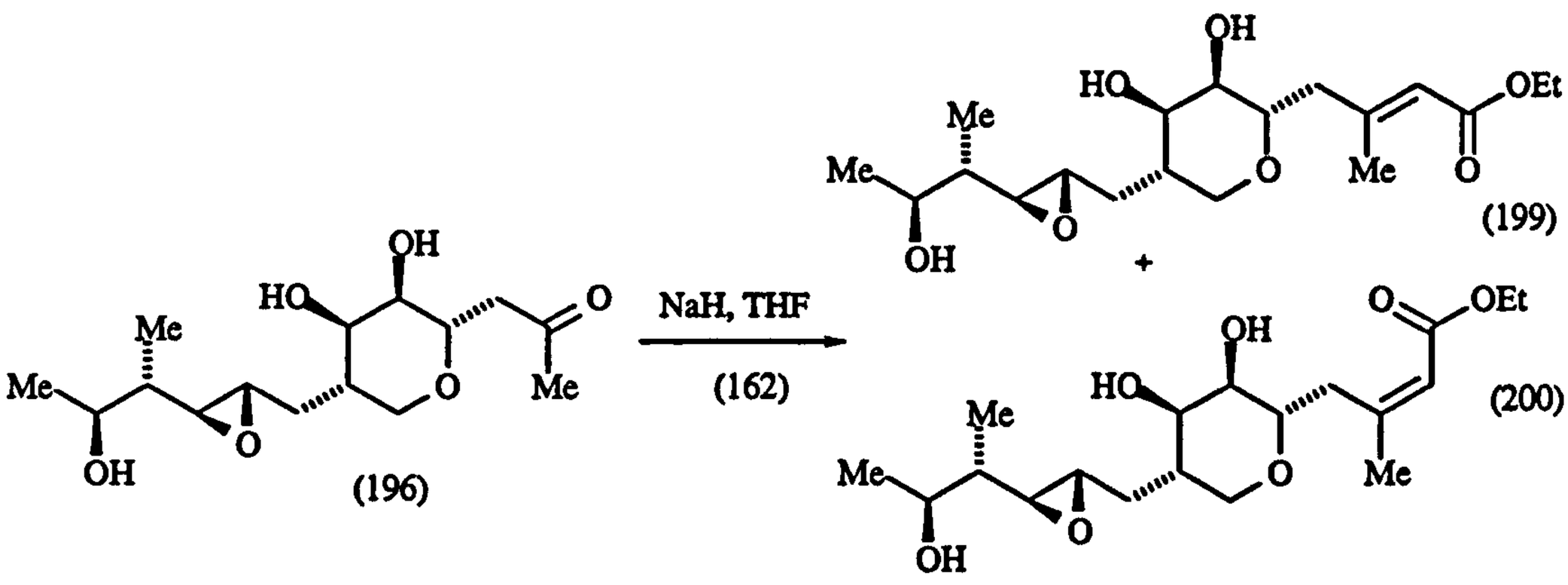
(196)

Alexander and coworkers[49] prepared the methyl ketone in quantitative yield, by ozonolysis of methyl pseudomonate (197), followed by reductive work up with triethylphosphite. Coulton and coworkers[118] later reported, however, that treating the methyl ketone with the stabilised Wittig reagent (198), resulted in no reaction.



(198)

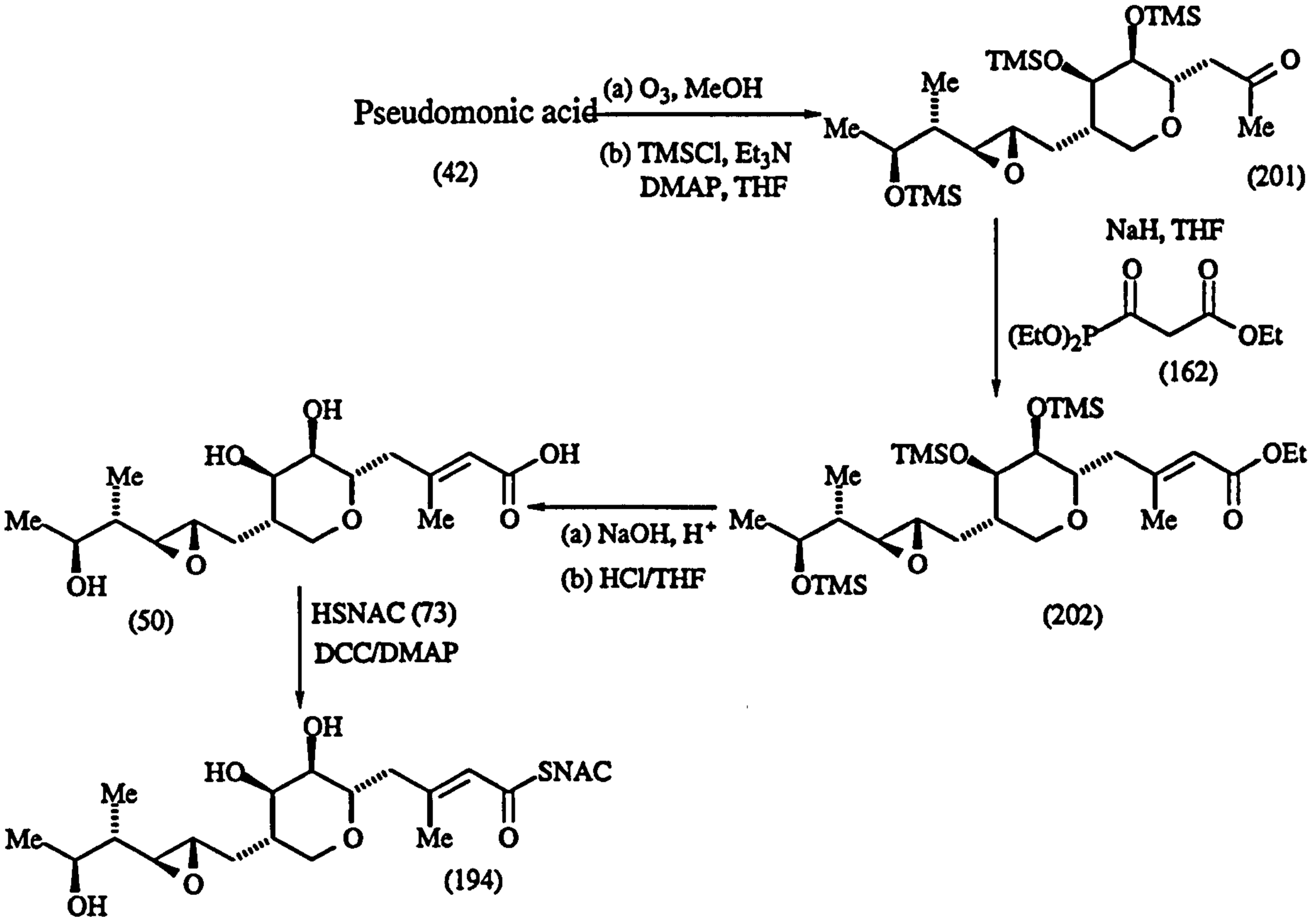
Reaction of the methyl ketone with the Horner-Wadsworth-Emmons reagent, ethyl diethylphosphonoacetate (162), in a stirred suspension of sodium hydride in THF, resulted in a complex mixture, in which ethyl monate (199) and ethyl isomonate (200) were obtained in a total yield of 15%, with a ratio of 3:1 (Scheme 2.48).[117]



Scheme 2.48: Synthesis of ethyl monate from the methyl ketone.

They showed that the total yield was improved to 80%, when the experiment was repeated with prior protection of the hydroxy groups in the methyl ketone as trimethylsilyl (TMS) ethers. Condensation of the TMS protected ketone (201) with ethyl diethylphosphonoacetate (162) produced the required (*E*) isomer (202) and the (*Z*) isomer (203) in a ratio of 3:1.[117]

This methodology was employed, in order to reach the target molecule, the NAC thioester (194) of monic acid A. Initially, the route below was attempted (Scheme 2.49)

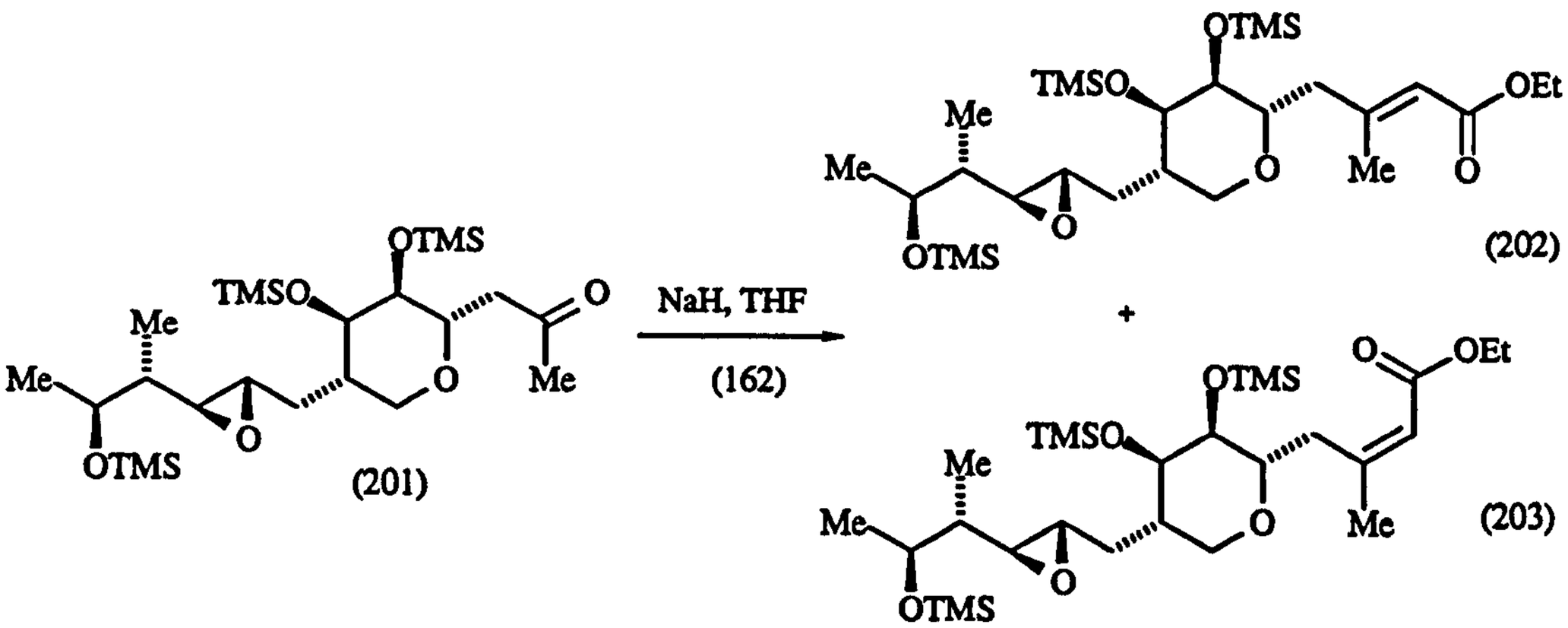


Scheme 2.49: Proposed approach for the synthesis of the NAC thioester of monic acid A.

Ozonolysis of methyl pseudomonate, in methanol, followed by reductive work up led to the formation of the methyl ketone (196). The ^1H nmr spectrum of (196) showed a characteristic singlet at 2.19 confirmed successful ozonolysis in 63% yield. Subsequent TMS protection was achieved, using the procedure of Crimmin and coworkers.[119] The methyl ketone, in THF, was treated with triethylamine, followed by trimethylsilylchloride (TMSCl) and a catalytic amount of DMAP led to the required product (201) in 70% yield. The sample was spectroscopically identical to an authentic sample supplied by P. J. O'Hanlon.[120]

Chain extension of (201) with ethyl diethyl phosphonoacetate (162), in a stirred suspension of sodium hydride in THF, gave (202). The optimum yields were obtained when 8 equivalents of sodium hydride and 10 equivalents of the phosphonoacetate were

used. The product was obtained in 30% yield. However, the ^{1}H nmr spectrum of the product indicated the presence of the required (*E*)-isomer (202) and the TMS protected ethyl isomonate (203) in a ratio of 4:1 respectively, which could not be separated. It has been shown that the natural (*E*) isomer of methyl pseudomonate can be distinguished from the (*Z*) isomer, methyl isopseudomonate by comparison of the vinylic methyl groups in both the ^{1}H and ^{13}C nmr spectra. A similar application was employed here to distinguish between the two monate esters. The ^{1}H nmr chemical shift of the vinylic methyl group in the TMS protected ethyl monate was δ 2.16, consistent with a deshielding effect of the *cis*-orientated ester group, and δ 1.98 in the TMS protected ethyl isomonate, in which the deshielding effect was absent. By measuring the integration of these two separate singlets, a ratio of *E:Z* of 4:1 was obtained (Scheme 2.50).



Ethyl monate (202): Ethyl isomonate (203) 4:1

Scheme 2.50: Synthesis of the TMS protected ethyl monate.

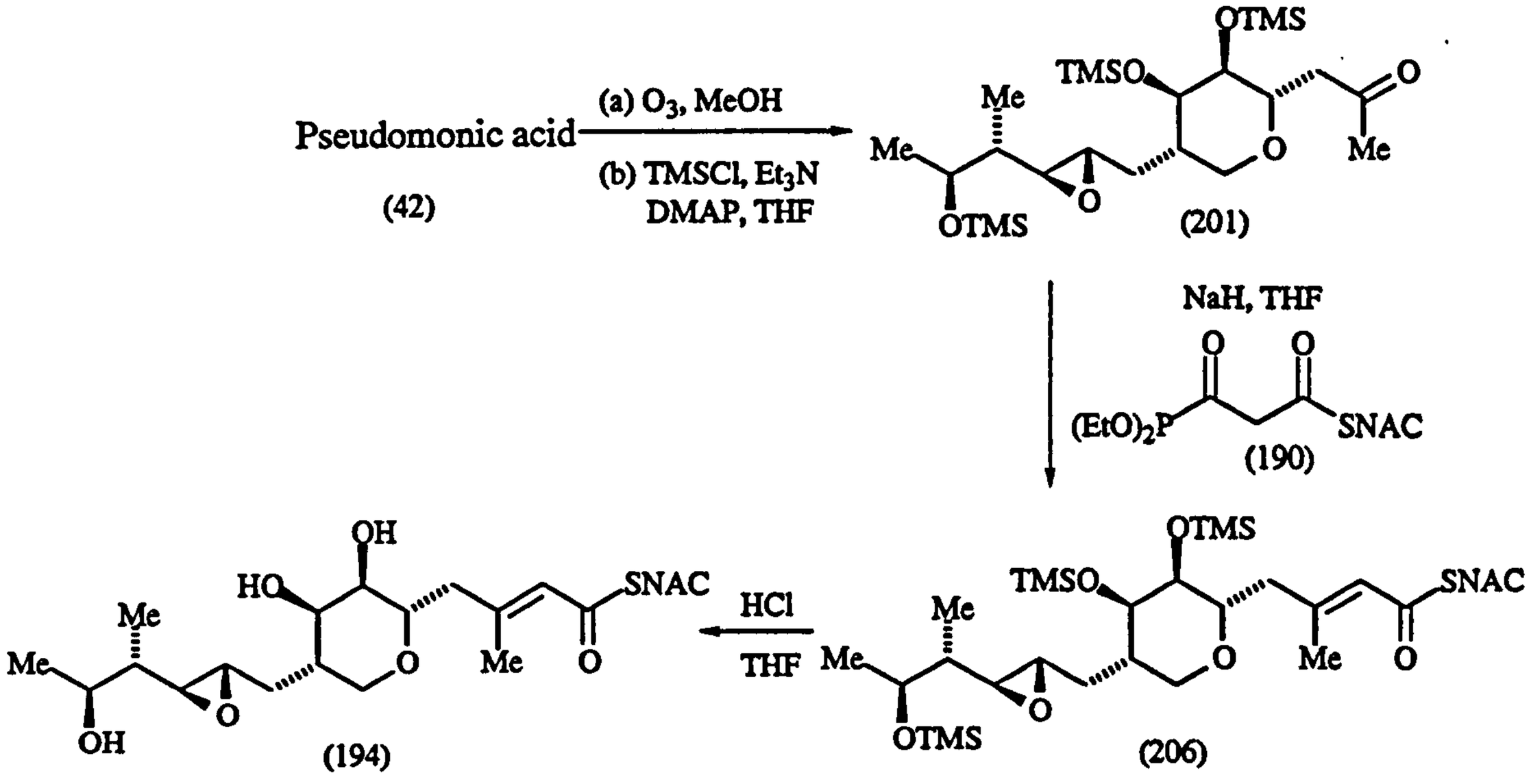
Treatment of the TMS protected ethyl monate (202) with sodium hydroxide solution gave, after acid work up, ethyl monate (204) in 85% yield. Similar results were obtained when using HCl/sodium hydrogen carbonate conditions. It is important that the experimental procedure is followed exactly, otherwise rearrangement products are likely to occur.

In an attempt to hydrolyse the ester, (204) was further treated with sodium hydroxide, but the rearrangement products (53) and (54) were isolated.[62] Neither monic acid A (50) nor the TMS protected monic acid A (205) were successfully prepared via this approach.

Monic acid A (50), supplied by SmithKline Beecham, has been shown to undergo DCC/DMAP mediated coupling with N-acetylcysteamine, leading to the formation of the NAC thioester (194) of monic acid A. Therefore, once the conversion problems of the conversion of TMS protected ethyl monate through to monic acid have been solved, the

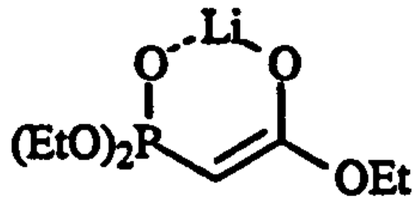
route will be complete, and may be applied to the synthesis of the NAC thioester of [1,2-$^{13}C_2$]monic acid A.

An alternative approach for the synthesis of the required thioester is shown in Scheme 2.51.



Scheme 2.51: Proposed synthesis of the NAC thioester of monic acid A.

Masamune and Roush[121] reported the use of lithium chloride, an amine, and ethyl diethylphosphonoacetate (162) to prepare α,β unsaturated esters in high yield, with an *E:Z* ratio of >50:1 with saturated aliphatic aldehydes, >20:1 with unsaturated aldehydes, and >100:1 with aromatic aldehydes. Their rationale behind this method was that lithium cations affect the course of Wittig and Horner-Wadsworth-Emmons reactions, by forming a tight complex with the carbanion derived from the phosphonate, thereby enhancing its acidity.



Treatment of the TMS protected ketone (199) with ethyl diethylphosphonoacetate and the TMS protected methyl ketone (201), under Masamune conditions, has proved unsuccessful. On work-up, the ^1H nmr spectrum showed only the presence of starting material. Similarly, treatment of the TMS protected ketone (201) with sodium hydride in THF and the NAC thioester of diethylphosphonoacetic acid (190) simply returned starting material. Once this step has been achieved, removal of the TMS groups, using HCl/sodium hydrogen carbonate is needed to reach the required thioester.

2.6 Conclusions and further work

The syntheses of a range of possible of isotopically labelled starter units of 9-hydroxynonanoic acid have been synthesised, including disodium malonate (97). Synthetic routes to sodium 3-hydroxypropionate (113) and the NAC thioester of 3-hydroxypropionic acid (99) have been developed. Work towards the synthesis of the NAC thioester of [1,2-$^{13}C_2$]-3-hydroxypropionic acid, utilising 2-oxazolidinones, is at present being completed.

A route has now been developed for the synthesis of the NAC thioester (137) of 9-hydroxynonanoic acid. The versatility of this route allows the synthesis of both the NAC thioesters of [1,2-$^{13}C_2$] and [2,3-$^{13}C_2$]-9-hydroxynonanoic acid. In addition, routes have been developed to the NAC thioester (169) of (2*S*,3*S*)-3-hydroxy-2-methylbutanoate, and to the NAC thioester (170) of (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-(2*E*)-enoic acid, required to investigate the biosynthesis of the monic acid moiety of pseudomonic acid. It is to be noted that the Baker's yeast reduction step was only effective in 70% enantiomeric excess. Thus, subsequent successful steps could only be assessed, based on a 70% e.e. starting material.

With reference to the synthesis of the NAC thioester of [1,2-$^{13}C_2$]monic acid A, the route is complete with the exception of the hydrolysis of the TMS protected ethyl monate A (202) to the TMS protected monic acid A (205). Once this route is complete, work can be directed towards the synthesis of the NAC thioester of [1,2-$^{13}C_2$]monic acid C. This requires conversion of the monic acid A to monic acid C, somewhere along the route, prior to the remaining steps to reach the thioester. There exist many methods for deoxygenating epoxides to produce olefins. For example, it is known that trifluoroacetyl iodide has been found to react with epoxides, in the presence of excess sodium iodide to produce related olefins in high yield.[122] The trifluoroacetyl iodide is generated *in situ* from trifluoroacetic anhydride and sodium iodide.

Chapter 3

Culture Work

3.1 Introduction

As described in chapter two, synthetic routes have now been developed to a number of putative biosynthetic precursors to pseudomonic acid (42). The next stage involves feeding these putative precursors, in isotopically labelled form, to *Pseudomonas fluorescens* to determine whether they are intermediates in the assembly of pseudomonic acid. Before incorporation experiments could be attempted, it was necessary to establish consistent growth conditions for *Pseudomonas fluorescens* NCIB 10586. For biosynthetic studies, a growth production curve needed to be determined in order to ascertain the optimum time to supplement the fermentation medium with isotopically labelled precursors. The following section will deal with each of the stages involved in establishing the production pattern of *Pseudomonas fluorescens*, and feeding studies with isotopically labelled putative biosynthetic intermediates.

3.2 Production of pseudomonic acid in *Pseudomonas fluorescens* NCIB 10586

The strain of *Pseudomonas fluorescens* originally selected for isolation studies was a soil isolate, and from this was developed a higher producing strain, *Pseudomonas fluorescens* NCIB 10586. This strain has been used for the majority of the work undertaken, except for a series of feeding studies carried out use the production strain *Pseudomonas fluorescens* PF3/R. The media used for growing *Pseudomonas fluorescens* were based on those developed by workers at SmithKline Beecham, and employed by Sugden.[77]

A lyophilised pseudomonic acid producing strain of *Pseudomonas fluorescens* NCIB 10586 was donated by SmithKline Beecham. This was used to produce agar slants, which would be viable for 3 months. Primary medium was inoculated with a 10µl loopful of *Pseudomonas fluorescens* grown on agar slants. After 24 hours at 25 °C, an aliquot was transferred to secondary medium, and grown for 120 hours. This secondary medium was glucose rich, thus enabling metabolite production to occur. The details of production of pseudomonic acid in *Pseudomonas fluorescens* are provided in the experimental section.

Pseudomonic acid production was monitored by HPLC. An aliquot (1ml) was taken from a flask, so as to determine the titre of pseudomonic acid present in the medium at that time. Examples of typical traces of the pseudomonic acid standards, and pseudomonic acid production during fermentation are shown below in Fig. 3.1 and Fig. 3.2 respectively.

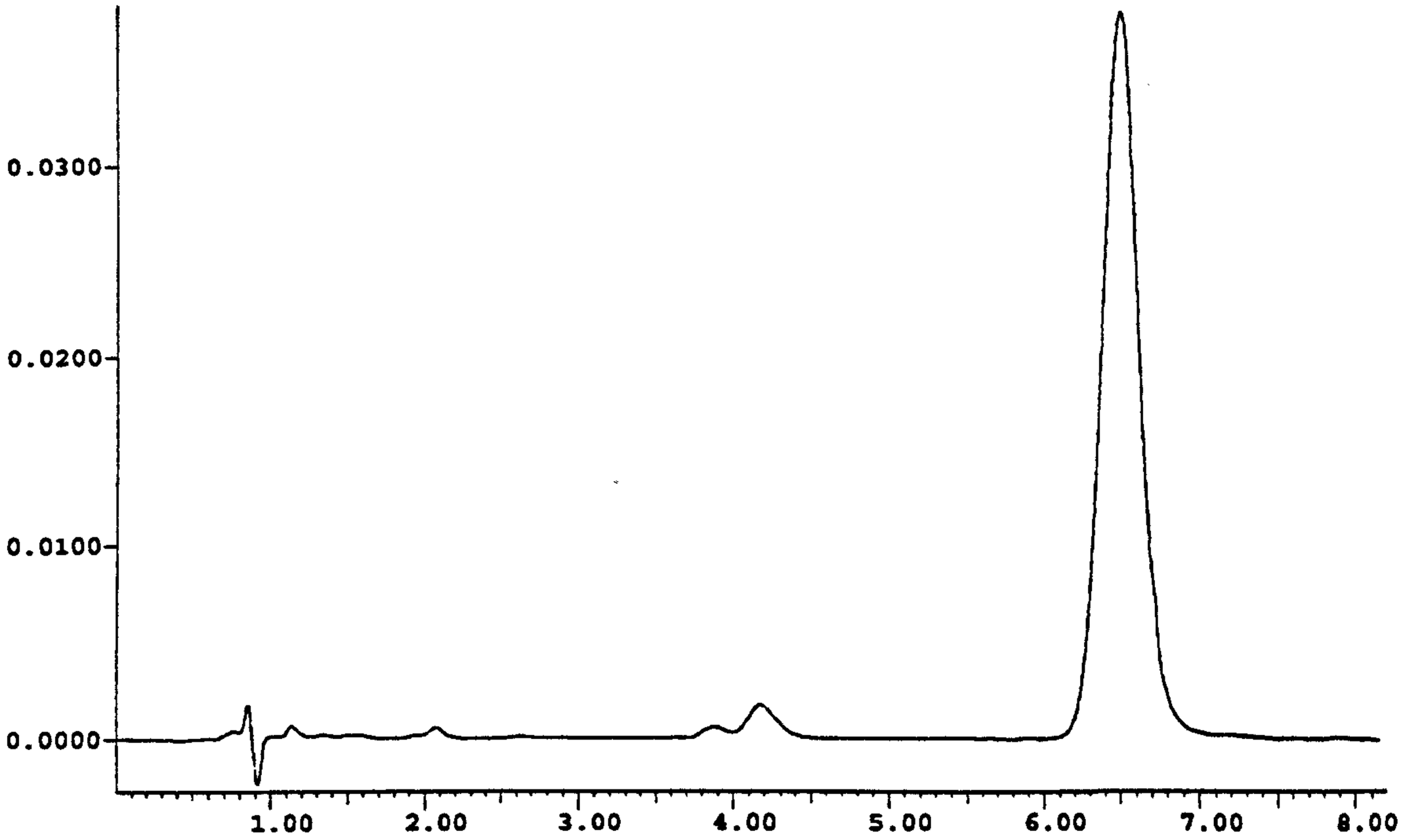


Fig. 3.1: HPLC trace of the pseudomonic acid standard.

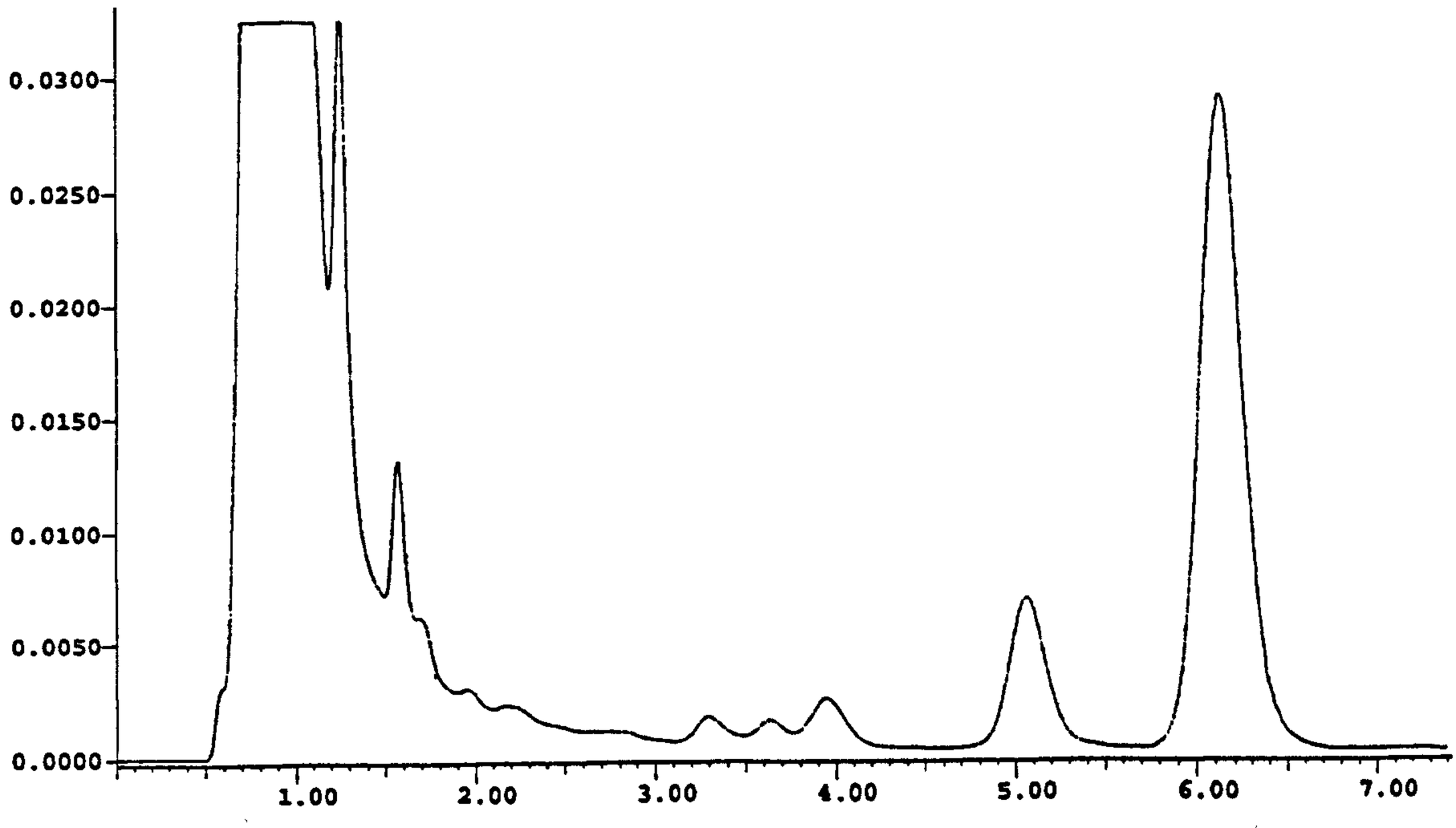


Fig. 3.2: HPLC trace of the pseudomonic acid production during fermentation.

For a growth production study, *Pseudomonas fluorescens* NCIB 10586 was grown in primary medium for 24 hours at 25 °C, and then an aliquot was transferred to secondary medium, where it was grown at 22 °C. An aliquot (1ml) was taken from at least two flasks every two hours, after a period of 10 hours after inoculation of the secondary medium, so as to determine the titre of pseudomonic acid present in the medium at that time. Each aliquot (1ml) was micro-centrifuged, and then diluted ten fold with distilled water. The samples were then either analysed immediately, or were stored at

-20 °C prior to analysis.

HPLC analysis enabled the growth production curve of pseudomonic acid to be constructed, as shown below in Fig. 3.3.

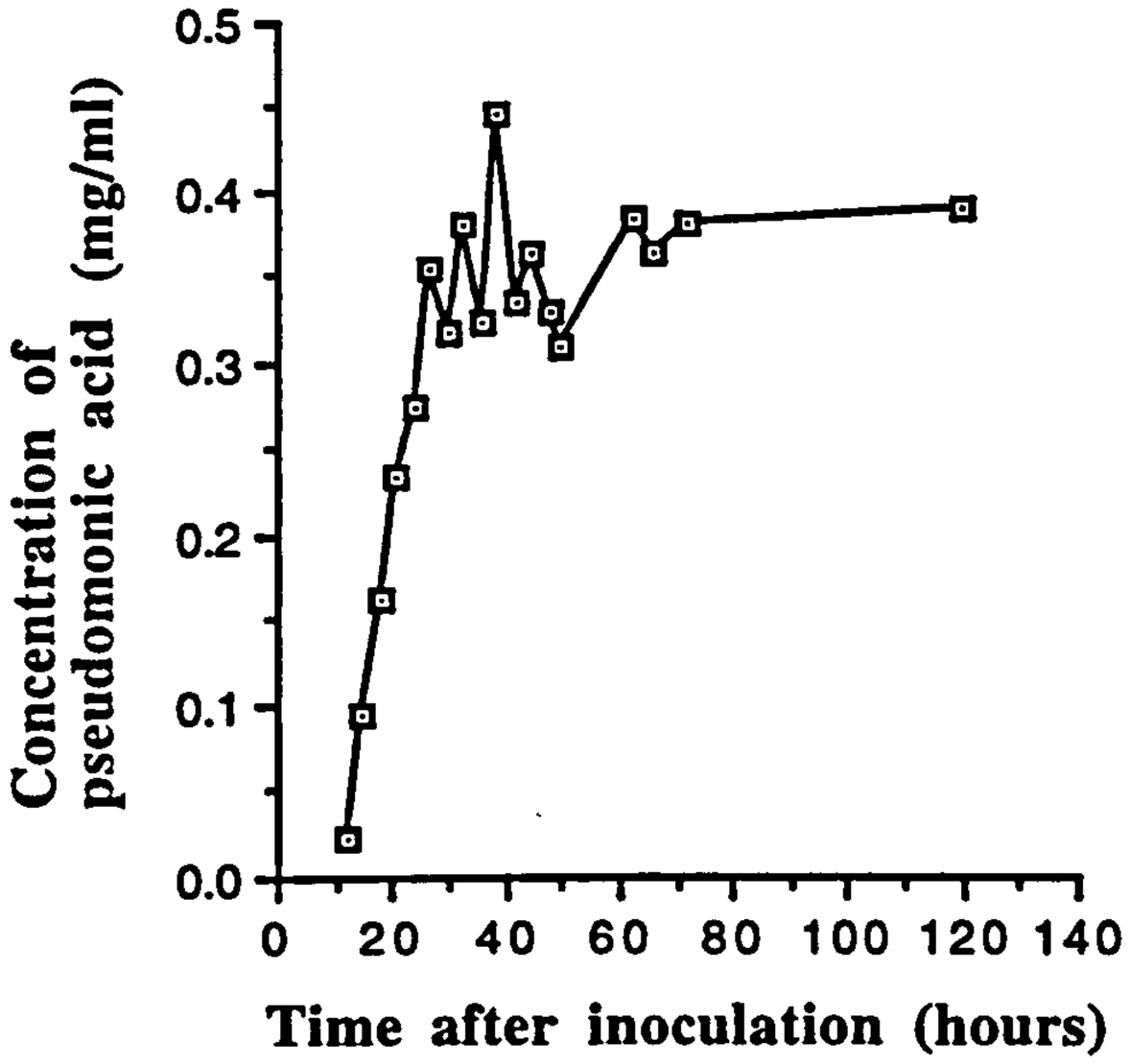


Fig. 3.3: Time course of pseudomonic acid production.

As shown in Fig. 3.3, pseudomonic acid production commenced about 12 hours after inoculation of the secondary medium. There then followed a rapid increase in pseudomonic acid production between 12 and 45 hours, which then reached a maximum concentration of 400mg/litre after about 50 hours.

In order to obtain the maximum incorporation of the isotopically labelled intermediate, this study indicated that these intermediates should be added to the fermentation medium at 12 hours, and isolation of pseudomonic acid should occur at 50 hours. However, it should be noted that in a typical 0.5 litre fermentation, the amount of isolated pseudomonic acid was considerably lower than that predicted by HPLC analysis. Typically, HPLC predicted a yield of 200mg of pseudomonic acid from a 0.5 litre fermentation. However, only 60mg, at most, of pseudomonic acid was ever isolated. This is probably due to the isolation procedure. In the isolation of pseudomonic acid, the combined secondary media was centrifuged, and the supernatant was then acidified to pH4.5, saturated with sodium chloride, and extracted with 500ml of ethyl acetate. However, during extraction, the formation of emulsions were a constant problem, and it usually took several hours for the extraction to be carried out. It was at this stage, that pseudomonic acid may have been lost.

The pH is also another important marker during pseudomonic acid production. An observed pH gradient decrease from pH 7.0 to 5.5 occurs whilst the pseudomonic acid

production increases. On reaching the maximum concentration, the pH then rises to 8.5, indicating the possible release of the enzyme bound pseudomonic acid. As previously shown in Scheme 1.18, pseudomonic acid rearranges at acid or alkaline pH, so that deviation from the observed pH curve in Fig. 3.4 will lead to a decrease in the amount of metabolite produced.

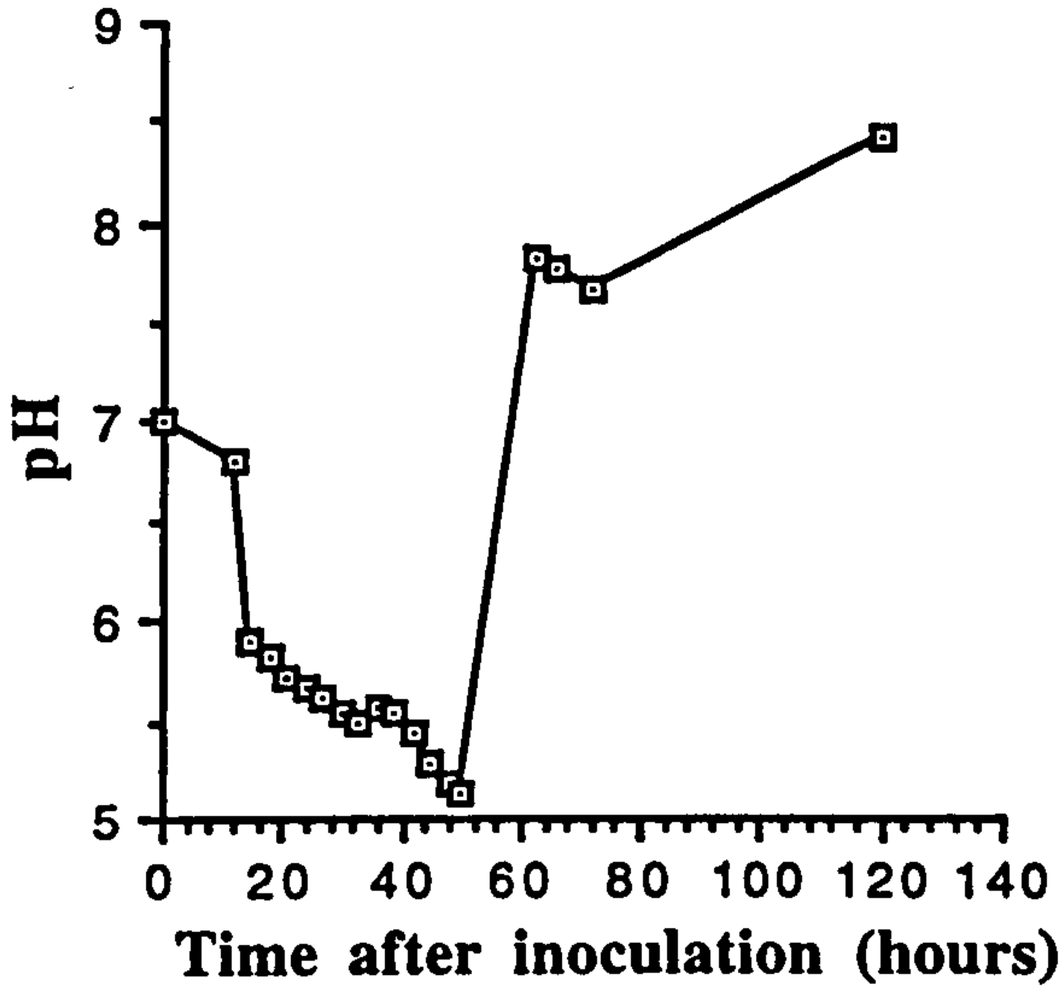
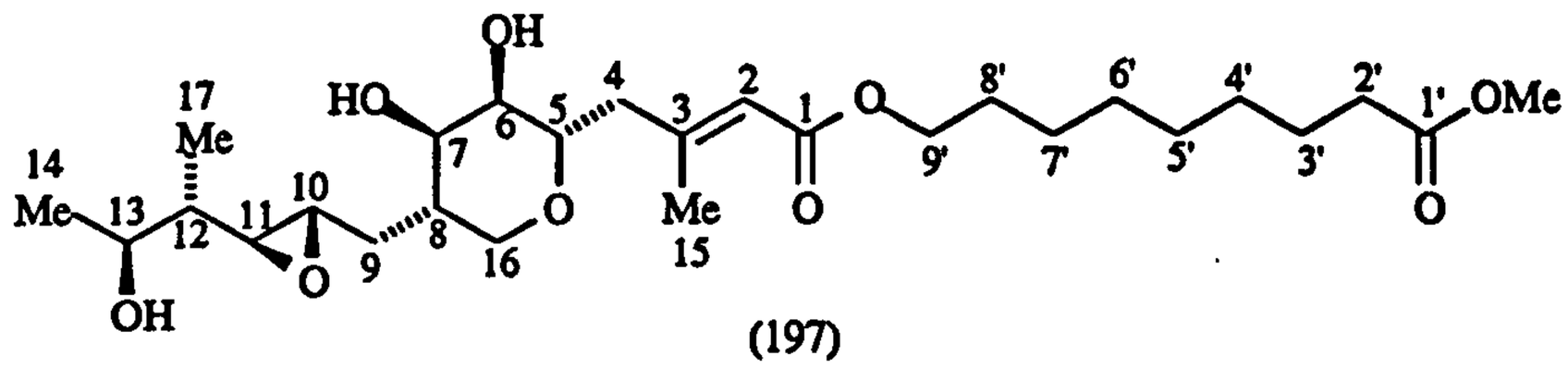


Fig. 3.4: Growth production curve, in terms of the pH of the fermentation broth.

On completion of the fermentation, pseudomonic acid was isolated, then methylated using diazomethane, and purified to give methyl pseudomonate (197).

The product was characterised by ^{1}H and ^{13}C nmr spectroscopy. The ^{1}H and ^{13}C nmr spectra were first assigned in 1977 by Mellows and coworkers,[65] and confirmed by Tyler and Everett in 1985.[123] Tables 3.1 and 3.2 show the ^{1}H and ^{13}C nmr spectral assignments, respectively, for an unlabelled, purified sample of methyl pseudomonate. The spectra are shown in Figures 3.5 and Fig. 3.6.



(197)

δ_H (ppm)	Multiplicity	J (Hz)	^{1}H Assignment
0.94	d	7.0	17-H
1.22	d	6.2	14-H
1.24-1.38	br s		4'-H, 5'-H, 6'-H, 7'-H, 12-H
1.46-1.98	br s		3'-H, 8'-H, 9-H
2.07	m		8-H
2.22	s		4ax-H, 15-H
2.31	t	7.2	2'-H
2.58-2.64	m		4eq-H
2.78-2.84	m		10-H
3.44-3.59	m		6-H, 16eq-H
3.67	s		OCH_3
3.72-3.96	m		5-H, 7-H, 13-H, 16ax-H
4.08	t	6.8	9'-H
5.76	s		2-H

Table 3.1: ^{1}H nmr spectral assignment of methyl pseudomonate.

δ_C (ppm)	^{13}C Assignment	δ_C (ppm)	^{13}C Assignment
12.7	C-17	51.4	OCH_3
19.0	C-15	55.5	C-10
20.7	C-14	61.3	C-11
24.9	C-3'	63.8	C-9'
25.9	C-7'	65.3	C-16
28.6	C-8'	68.9	C-6
29.0	C-4'	70.3	C-7
29.1	C-5', C-6'	71.4	C-13
31.5	C-9	74.8	C-5
34.1	C-2'	117.6	C-2
39.4	C-8	156.6	C-3
42.8	C-4	166.8	C-1
42.8	C-12	174.4	C-1'

Table 3.2: ^{13}C nmr spectral assignment of methyl pseudomonate.

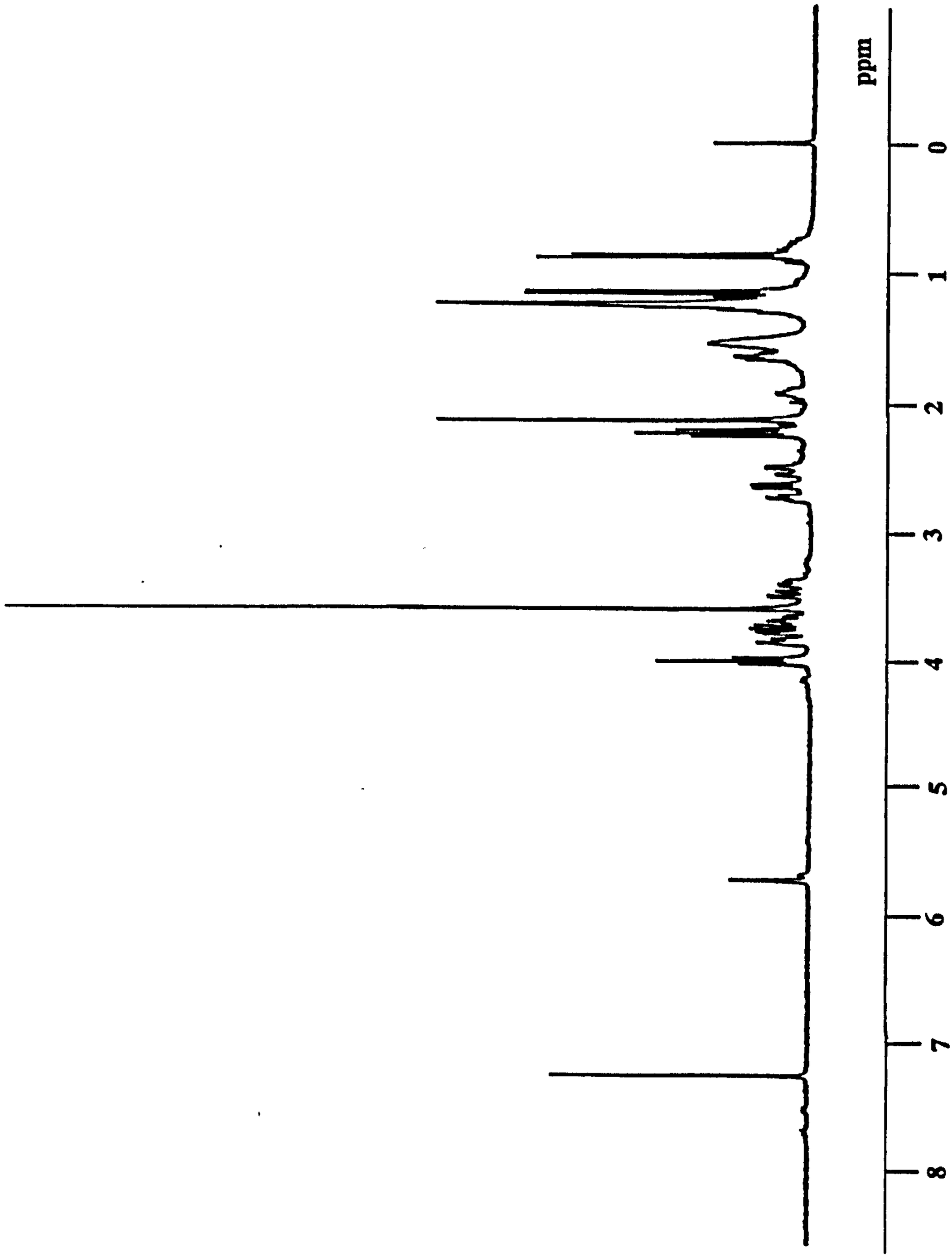


Fig. 3.5: 270 MHz ^{1}H nmr spectrum of methyl pseudomonate in $CDCl_3$.

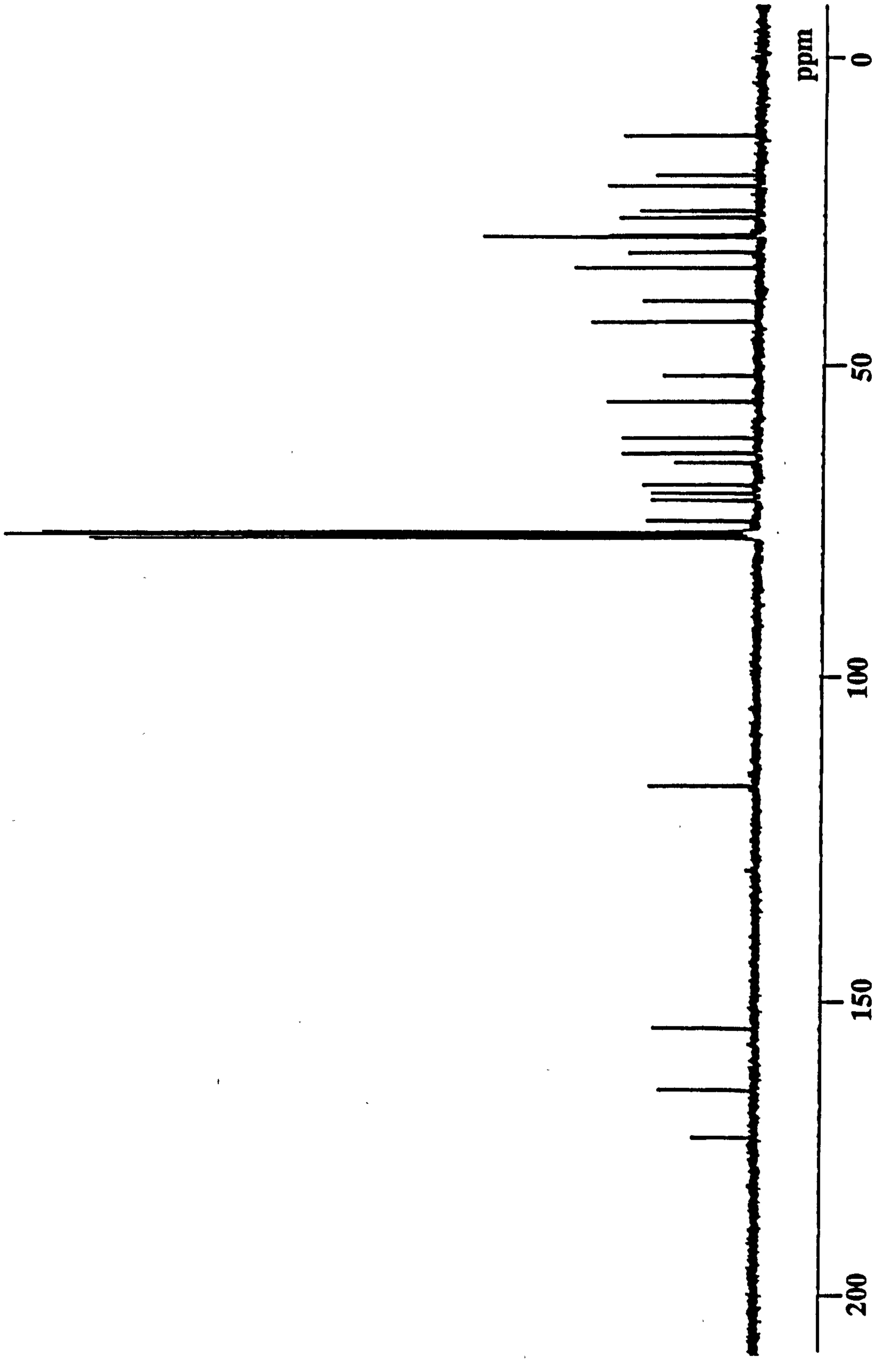


Fig. 3.6: 270 MHz ^{13}C nmr spectrum of methyl pseudomonate in $CDCl_3$.

3.3 Incorporation studies with *Pseudomonas fluorescens* PF3/R and NCIB 10586

A growth production study was carried out similarly using *Pseudomonas fluorescens* PF3/R. Pseudomonic acid production was slower than with NCIB 10586, and was found to reach a maximum after 7 days. The study indicated the best time to supplement the fermentation was at 43 hours, and that for maximum incorporation of labelled substrates, isolation should take place at 144 hours.

Eight feeding studies were carried out using PF3/R. The eight substrates were sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$]acetate, sodium [1-^{13}C]propionate, sodium [2-^{13}C]propionate, sodium [3-^{13}C]propionate, sodium [1-^{13}C]butyrate, and disodium [1,2-$^{13}C_2$]malonate. In each case, the substrate was fed after 43 hours of inoculation of the final stage medium, a stage which HPLC analysis had shown a value of 11% of the value of the control medium at 144 hours.

However, after leaving the flasks shaking at 22 °C for 6 days, the pH was 5.5, instead of the expected pH 8.0. In order to release the pseudomonic acid that had been produced, 150µl of 8M KOH was added to adjust the pH to 8.0. A HPLC assay was then carried out on each feed. However, for the acetate feeds HPLC gave a value of 12%, for the propionate feeds HPLC gave a value of 14%, and for the butyrate feed HPLC gave a value of 15% of the value of the control medium at 144 hours.

Hence these results show that on feeding the labelled substrates, no more pseudomonic acid was produced, and hence no significant incorporation was achieved. This was verified by isolating pseudomonic acid, converting to the methyl ester by reacting it with an excess of diazomethane, and purified by preparative TLC. Low yields of methyl pseudomonate, which equated to the pseudomonic acid present before the labelled substrates were fed. This process was repeated for each feed, after which ^{1}H and ^{13}C nmr spectra were obtained for each feed. In each case, the ^{13}C nmr spectrum of methyl pseudomonate showed no significant incorporation of the labelled substrate.

Pseudomonas fluorescens PF3/R is a much higher producing engineered strain, in which feeding labelled substrates appears to have halted the further production of pseudomonic acid. Subsequent studies were carried out with the lower producing strain of *Pseudomonas fluorescens* NCIB 10586.

The growth production study was repeated with *Pseudomonas fluorescens* NCIB 10586, with the addition of unlabelled sodium acetate. The pH was monitored every three hours to see if the pH remained at 4.5, and then increased to pH 8.0 at 120 hours, as expected, or whether the pH of the broth remained low. In fact, the pH was found to stay at 4.5, once pseudomonic acid had commenced at 12 hours.

Two sets of experiments were then carried out. One experiment involved ten flasks being inoculated with sodium [1-^{13}C]acetate as a single feed at 15 hours (at a level of 1mg/ml); the other in which ten flasks were inoculated with a pulse feed at 14,16,18

hours (at a level of 0.4 mg/ml, 0.4 mg/ml, and 0.3 mg/ml). After 50 hours all the flasks were harvested. The pH of the combined broths of the single feed was 4.5, as was the pH of the pulse feed broths, indicating inhibition of pseudomonic acid production. HPLC assay confirmed very little pseudomonic acid production had been produced. The pulse feed produced 4mg of pseudomonic acid, compared with 2 mg of pseudomonic acid with the single feed. However, the control flasks produced 30mg of pseudomonic acid, so the acetate is inhibiting production of pseudomonic acid.

Feeding studies with sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$]acetate, sodium [1-^{13}C]propionate, sodium [2-^{13}C]propionate, sodium [3-^{13}C]propionate, and sodium [1-^{13}C]butyrate were also carried out with the NCIB 10586 strain. Analogous results were obtained with NCIB 10586, as with PF3/R, with there being inhibition of pseudomonic acid production to those flasks, which were fed with labelled substrates. In each case, very low yields of methyl pseudomonate were isolated, from which unsatisfactory ^{13}C nmr spectra were obtained.

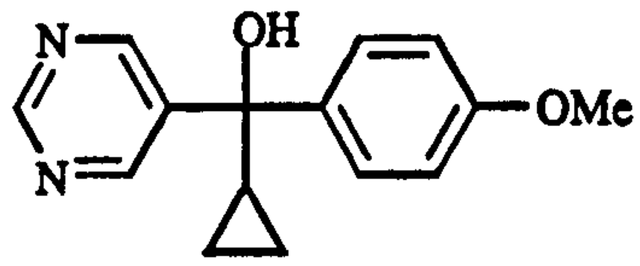
Feeding labelled substrates to P*seudomonas fluorescens* PF3/R and NCIB 10586 has been shown to inhibit the production of pseudomonic acid, under the present conditions. A possible explanation for these results is that essentially the labelled substrates are acid buffers, and as a result inhibit or stop producing pseudomonic acid when in acidic conditions. One way to avoid this problem may be to buffer the system, so that on addition of the labelled substrate there is not such a sharp drop in the pH of the broth.

With all these feeding experiments, either 100 mg or 200 mg of the labelled substrate have been added. Perhaps this is far in excess of what *Pseudomonas fluorescens* can cope with. It may be worth attempting to feed smaller quantities of the labelled substrate. Pulse feeding the labelled substrate has also shown that there is less inhibition of pseudomonic acid production, compared with a single feed of the labelled substrate. For future incorporation studies, pulse feeding, as opposed to a single feed, would be recommended.

Finally, it may be worth reverting to the initial conditions employed by Mellows and coworkers for future studies.[65] This will entail changing the constituents of the primary and secondary media, establishing a new growth production curve, and then carrying out preliminary [1-^{13}C]acetate feeds, before utilising the putative precursors to pseudomonic acid in future incorporation studies.

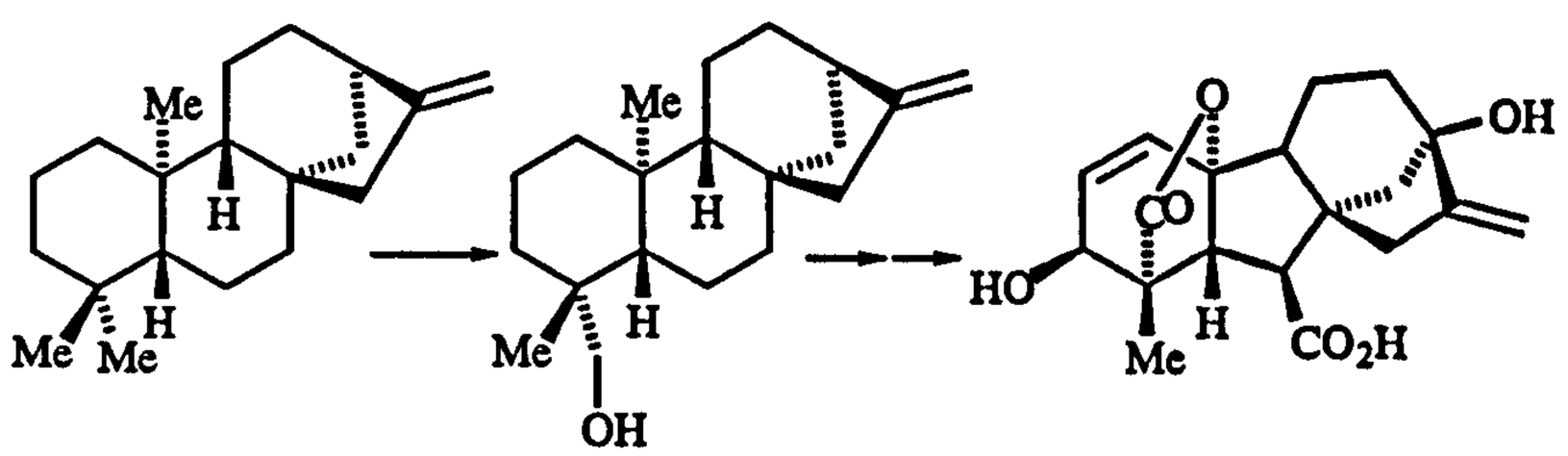
3.4 Enzyme inhibitor studies

Cytochrome P_{450} is an oxidase present in numerous organisms from bacteria to mammals, and plays an important role in the biosynthesis of sterols and the gibberellin plant hormones. Oxidase inhibition been successfully used in the elucidation of biosynthetic pathways of many secondary metabolites, such as rifamycin[124] and erythromycin.[125] A known inhibitor of these monooxygenases is ancymidol (207).[126,127]



(207)

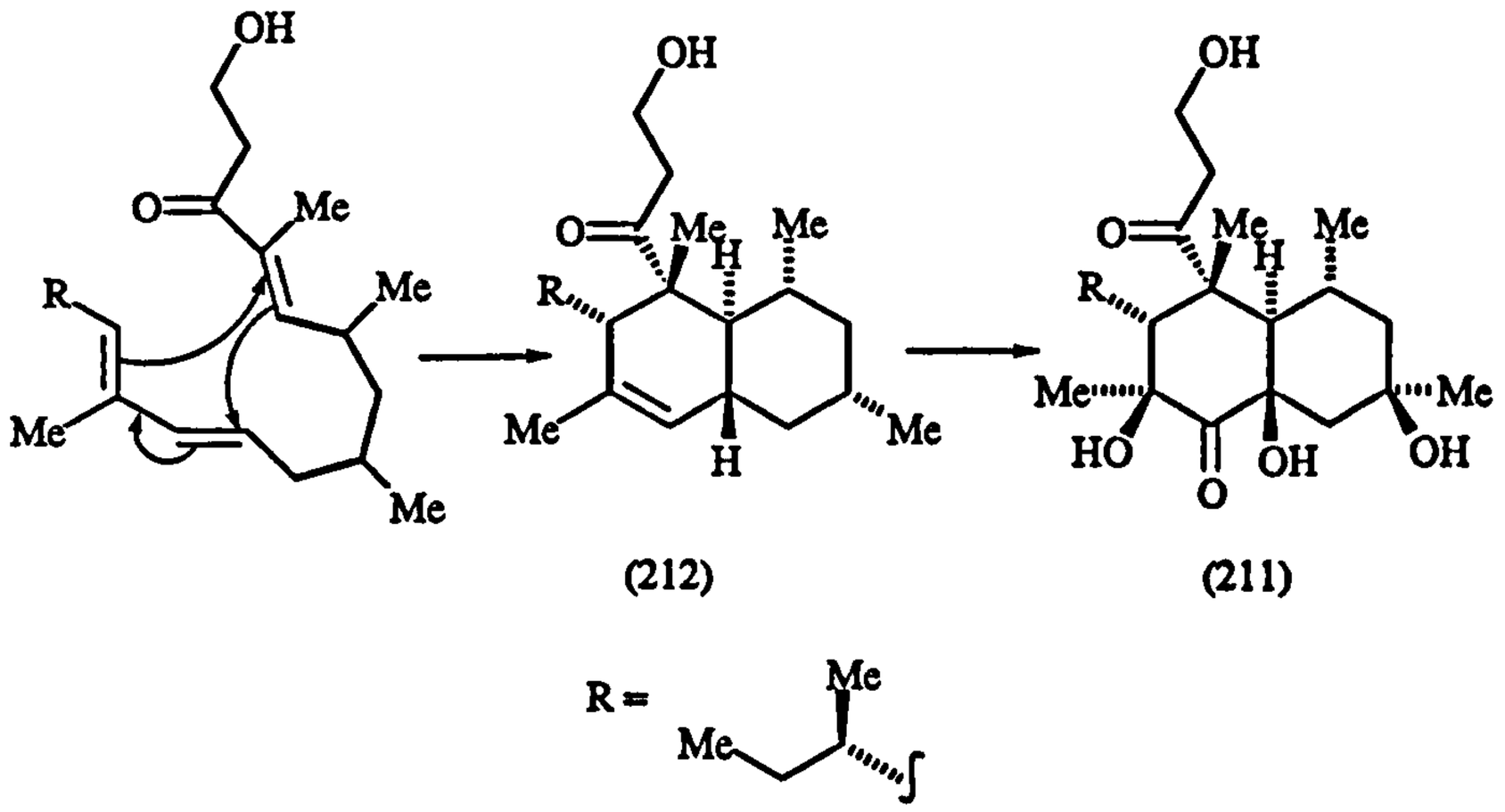
This inhibitor has been shown to block the conversion of kaurene (208) to kaurenol (209) on the gibberellic acid (210) biosynthetic pathway, as outlined in Scheme 3.1.[128]



(208) (209) (210)

Scheme 3.1: The oxidation of kaurene to kaurenol; a biosynthetic step inhibited by ancymidol.

Ancymidol has also been applied to the study of betaenone B (211) biosynthesis, a polyketide metabolite produced by the fungus *Phoma betae*. Betaenone B is formed via the condensation of acetate with eight malonate units, with the branching methylation resulting from methionine.[129] The use of [1-^{13}C, $^{18}O_2$]acetate indicated that the ketone oxygen at C-1 was not acetate derived. From this, a biogenetic hypothesis involving an intramolecular Diels-Alder reaction was postulated (Scheme 3.2).[130]

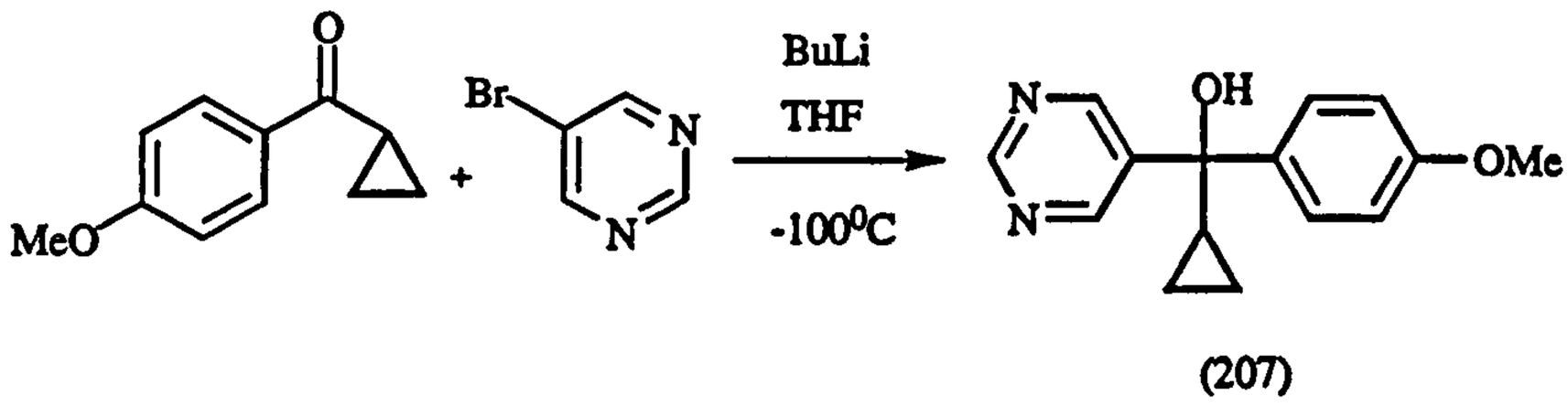


Scheme 3.2: The biosynthesis of betaenone B.

Oikawa and coworkers then showed that, by using ancymidol as a monooxygenase inhibitor, betaenone B production was suppressed. The reduction in titre was directly proportional to the amount of ancymidol administered, and a new metabolite was found to accumulate, which was shown to be the intermediate (212) proposed as the initial Diels-Alder product.[130] This information provided further evidence that such a biosynthetic pathway towards betaenone B was in operation.

Martin and Simpson[71,72] showed that pseudomonic acid has two oxygen atoms which are not derived from acetate. These are the epoxide oxygen, and the hydroxyl on C-6. These are assumed to originate from atmospheric oxygen, although experiments designed to confirm this, by using an $^{18}O_2$ enriched atmosphere, did not produce enough metabolite for any deductions to be made. If this assumption is correct, the oxidase involved is likely to be a cytochrome P_{450} type enzyme.[131]

Hence, our aim was to carry out a similar experiment to that with betaenone B, so as to measure the effect of ancymidol on pseudomonic acid biosynthesis. Due to the expense of commercially available ancymidol, it was prepared by Dickinson,[132] according to the procedure of Davenport (Scheme 3.3).[133]



Scheme 3.3: Synthesis of ancymidol.

To each flask of secondary medium, inoculated with *Pseudomonas fluorescens* NCIB 10586 was added 0 mg, 5 mg, 10 mg, 25 mg, 50 mg, and 100 mg of ancymidol at

the start of pseudomonic acid production at 15 hours. The cultures were then left to grow for 5 days, before HPLC analysis. No significant difference in the titre of pseudomonic acid was found, compared with the control flasks. No new metabolites were observed by HPLC.

3.4 Conclusions and future work

In conclusion, synthetic routes have been developed to a series of putative precursors of pseudomonic acid. In order to investigate the role of 9-hydroxynonanoic acid biosynthesis, synthetic routes have been developed to various forms of the two possible starter units of 9-hydroxynonanoic acid: malonate (97) and 3-hydroxypropionate (113). Also, a synthetic route to the NAC thioester (137) of 9-hydroxynonanoic acid was required, and has been completed. The versatility of this synthesis allows both the [1,2-$^{13}C_2$] and [2,3-$^{13}C_2$] isotopically labelled forms to be prepared, making use of the Horner-Wadsworth-Emmons methodology in coupling a C_7 aldehyde and a C_2 phosphonate.

Synthetic routes have also been developed to the diketide (169) and triketide (170) putative intermediates of monic acid. The development of a route enabling the synthesis of the NAC thioester of [1,2-$^{13}C_2$]monic acid A has been undertaken, but still requires completion.

To date, several initial feeding studies have been carried out. Further work needs to be carried out in this area, to investigate why the growth of *Pseudomonas fluorescens* is inhibited on feeding precursors to the fermentation broth. Only when these problems are solved, can other putative intermediates, including the proposed later stage precursors, be fed with the hope of elucidating more information on the biosynthetic pathway to pseudomonic acid.

Chapter 4

Experimental

4.1 General experimental details

(i) Techniques

Unless otherwise stated, all non-aqueous reactions were carried out under a nitrogen atmosphere, and all solvents were purified according to literature procedures.[134] Nitrogen was dried by passage through a silica gel / calcium chloride column.

Preparative Thin Layer Chromatography

Preparative thin layer chromatography was carried out on 200 x 200 mm glass plates coated with silica gel (0.25, 0.5 or 1cm, Fluka 60765, Kieselgel G_f245).

Flash Column Chromatography

Flash column chromatography was carried out according to the procedure of Still *et al* [135] using Fluka silica gel 60 (70638) as the solid support. Compounds were loaded in the minimum amount of ethyl acetate, and eluted with mixtures of ethyl acetate and 40-60 °C petroleum ether of varying polarity.

Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out using Merck Kieselgel 60 glass backed TLC plates, with various ethyl acetate and 40-60 °C petroleum ether mixtures used as an eluent. Bands were visualised by using one of the following methods:

i) fluorescence on exposure to short wave ultra-violet light (245 nm)

ii) by developing in an ammonium molybdate (10% w/v in 2M sulphuric acid), followed by heating with a hot air gun

iii) by developing in an aqueous potassium permanganate (0.5%) and sodium hydrogen carbonate (2.5%) solution, followed by heating with a hot air gun.

Microbiological work

At Bristol, microbiological work was performed under sterile conditions, in a Biomat Class II microbiological cabinet. At Worthing, the work was carried out in an open plan sterile air flow room. Sterilisation of all materials prior and subsequent to use was carried out in an autoclave under the described conditions.

For growth of *Pseudomonas fluorescens*, the constituents of the primary and secondary media are commercially available. Agar No3 was obtained from Oxoid. Spray dried corn steep liquor, antifoam, and spray dried yeatex were obtained from Sigma.

(ii) Instrumentation

Nuclear Magnetic Resonance (NMR)

^{1}H and ^{13}C nmr spectra were recorded as solutions in deuterochloroform, unless otherwise stated, using $CDCl_3$ as a reference to δ_H 7.25 and δ_C 77.0 respectively. The spectra were recorded on a Jeol GX270 or a GX400 spectrometer. Those spectra run in $^{2}H_2O$ were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulphonate. The chemical shift values for all spectra are given in parts per million with the coupling constants in Hertz (Hz).

Mass Spectrometry

Low and high resolution mass spectra were recorded on a Fisons Autospec spectrometer. Spectra were obtained using electronic ionisation (EI) (at a potential of 70eV) or chemical ionisation (CI) techniques. Data given is for EI, except were indicated.

Infra Red Spectroscopy

Spectra were recorded on a Perkin Elmer 881 infra red spectrometer as thin films, except were indicated.

Optical rotations

Optical rotations were measured using the sodium-D line on a Perkin-Elmer 241MC polarimeter as solutions in chloroform, except were indicated.

Melting points

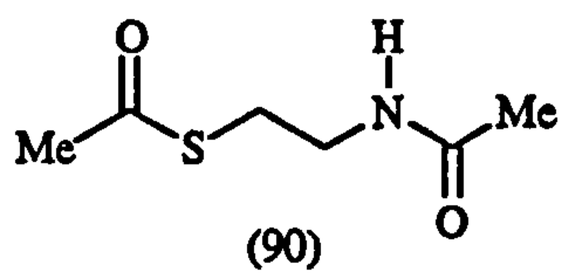
Melting points were determined on a Gallenkamp Melting Point apparatus, and are uncorrected.

High Pressure Liquid Chromatography (HPLC)

At Bristol, HPLC was carried out using a Gilson 303 system. At Worthing, HPLC was carried out using a Gilson apparatus. In both cases, a 12.5cm Whatman Perisphere C-18 (10U packing) was used. The mobile phase was 20% methanol, 80% water, acidified with ammonium acetate at 0.58 g/l. A flow rate of 1.5 cm^3 min^{-1} was used, with the peaks being detected at 233nm.

4.2 Experimental for the syntheses discussed in Chapter 2

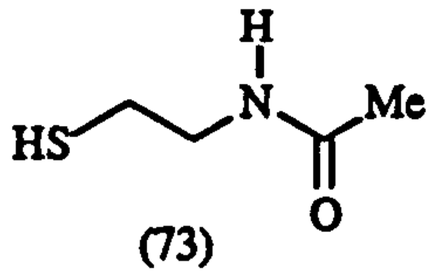
S-2-(Acetylamino)ethyl ethanethioate (90) (N,S-Diacetylcysteamine)[86]



(90)

To a 3-necked flask at 0 °C containing 2-mercaptoethylamine hydrogen chloride (89) (5.76 g, 50.7 mmol) in water (50 cm^3) was attached two dropping funnels and a pH probe. In one funnel was placed potassium hydroxide (8M, 50 cm^3), and in the other was placed acetic anhydride (14.5 cm^3). These were both then added dropwise at such a rate that the pH was maintained at 8.0. After all the acetic anhydride was added, the pH was adjusted to 7.0 with 2M HCl, and the mixture was stirred at room temperature for 1 h.. The reaction was saturated with sodium chloride, and was extracted with dichloromethane (4 x 50 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield S-2-(acetylamino)ethyl ethanethioate (90) as a colourless oil (8.1 g, 99%), which solidified upon refrigeration. υ_{max} 1655 cm^{-1}; δ_H 1.99 (3H, s, C$\underline{H}_3$COS), 2.36 (3H, s, C$\underline{H}_3$CON), 3.03 (2H, t, *J* 6.5, C$\underline{H}_2$S), 3.41 (2H, t, *J* 6.5, C$\underline{H}_2$N), 6.66 (1H, br s, N$\underline{H}$); δ_C 22.81 ($\underline{C}H_2S$), 28.46 ($\underline{C}H_2NH$), 30.39 ($\underline{C}H_3COS$), 39.16 ($\underline{C}H_3CON$), 170.50 ($\underline{C}ON$), 195.99 ($\underline{C}OS$); *m/z* 161 (M^+, 0.2%), 119 (60), 118 (21), 86 (9), 72 (19), 60 (30), and 43 (100).

S-2-(Acetylamino)ethanethiol (73) (N-Acetylcysteamine)[86]

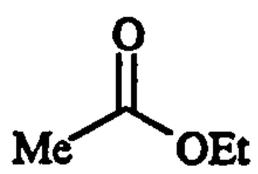


(73)

N,S-Diacetylcysteamine (90) (5.3 g, 32.9 mmol) in water (80 cm^3) was cooled to 10 °C. Solid potassium hydroxide (5.57 g, 99.5 mmol) was added, and the reaction was stirred, under a nitrogen atmosphere, at 10 °C for 50 minutes. The pH was adjusted to pH 7 with hydrochloric acid (2M), and the reaction was saturated with sodium chloride and extracted with dichloromethane (10 x 50 cm^3). The organic extracts were dried over magnesium sulphate, and concentrated *in vacuo* to yield S-2-(acetylamino)ethanethiol (73) (3.63 g, 93%) as a colourless oil. υ_{max} (thin film) 2547, 1650, 1562 cm^{-1}; δ_H 1.41 (1H, t, *J* 8.5, S$\underline{H}$), 2.00 (3H, s, C$\underline{H}_3$CON), 2.67 (2H, dt, *J* 8.5, 6.5, C$\underline{H}_2$S), 3.42 (2H, q, *J* 6.5, C$\underline{H}_2$N), 6.40 (1H, br s, N$\underline{H}$); δ_C 23.03 ($\underline{C}H_2N$), 24.24 ($\underline{C}H_2S$), 42.30

($\underline{C}H_3CO$), 170.37 ($CH_3\underline{C}O$); *m/z* 119 (M^+, 29%), 118 (5), 86 (22), 72 (31), and 60 (100).

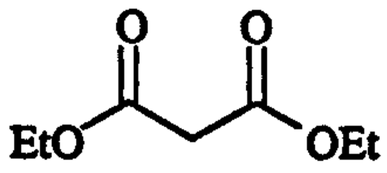
Ethyl acetate (101)[91]



(101)

To anhydrous sodium acetate (100) (1.00 g, 12.20 mmol) was added triethylphosphate (5 cm^3), and the reaction mixture was heated to reflux for three hours. After cooling in an ice-bath for 0.5 h., the condenser was linked to a vacuum pump *via* two traps. The first of these was cooled with ice/salt, whilst the second was cooled with liquid nitrogen. The system was evacuated, and the reaction mixture warmed, so ethyl acetate collected in the second trap, whilst any triethylphosphate collected in the first trap. This yielded ethyl acetate (101) (0.74 g, 70%) as a colourless oil. υ_{max} 1742 cm^{-1}; δ_H 1.26 (3H, t, *J* 7.0, $C\underline{H}_3CH_2$), 2.05 (3H, s, $C\underline{H}_3CO$), 4.12 (2H, q, *J* 7.0, $CH_3C\underline{H}_2O$).

Diethyl malonate (102)[77]

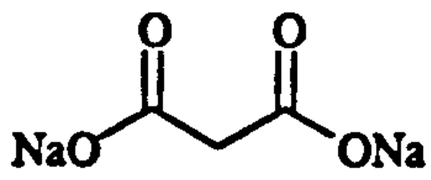


(102)

To THF (20 cm^3) at -78 °C under a dry nitrogen atmosphere was added hexamethyldisilazide (2.04 g, 12.64 mmol). To this was added n-butyl lithium (2.5M in hexanes, 5 cm^3, 12.50 mmol). The stirred solution was then warmed to 0 °C, and then cooled again to -78 °C. After 0.5 h., ethyl acetate (101) (0.74 g, 8.41 mmol) was added dropwise as a solution in THF (3 cm^3). After 40 minutes, ethyl chloroformate (1.37 g, 12.63 mmol) was added. The resulting solution was then left for 3 hours, after which it was quenched with HCl (2M, 15 cm^3). The THF was removed *in vacuo* to leave a pale orange oil. The residue was then extracted with diethyl ether (3 x 50 cm^3) and HCl (2M, 40 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered, and the solvent was removed *in vacuo*. Purification by Kugelrohr distillation (66 °C, 6 mmHg) yielded diethyl malonate (102) (0.76 g, 57%) as a colourless oil. lit.,[77] bp. 66 °C at 6 mmHg; υ_{max} 1759 cm^{-1}; δ_H 1.25 (6H, t, *J* 6.2, 2 x $C\underline{H}_3CH_2O$), 3.37 (2H, s, $C\underline{H}_2$), 4.21 (4H, q, *J* 6.2, 2 x $CH_3C\underline{H}_2O$); δ_C 13.98 ($\underline{C}H_3CH_2O$), 41.63 ($\underline{C}H_2(CO_2Et)_2$), 61.44 ($CH_3\underline{C}H_2O$), 166.59 ($\underline{C}O_2Et$); *m/z* 160 ($M^+$, 22%), 115 (39), 87 (26), 43 (43),

and 29 (100).

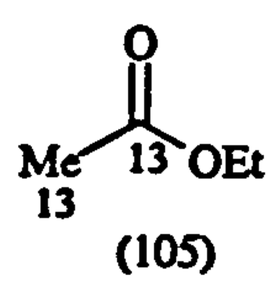
Disodium malonate (97)[77]



(97)

To diethyl malonate (102) (0.68 g, 4.25 mmol) was added 2M sodium hydroxide solution (4.25 cm^3, 8.5 mmol). The mixture was stirred at room temperature for 18 hours. The water was removed *in vacuo*. The remaining water was then removed by freeze-drying giving disodium malonate (97) (0.72 g, 92%) as a white crystalline solid. mp. >270^{0}C; υ_{max} (nujol) 1580 cm^{-1}; δ_H (^2H_2O) 3.12 (2H, s, $C\underline{H}_2$); δ_C (^2H_2O) 47.30 ($\underline{C}H_2OH$), 177.41 ($\underline{C}O_2Na$).

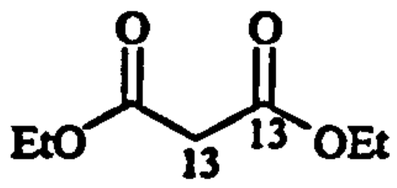
Ethyl [1,2-$^{13}C_2$]acetate (105)[77]



(105)

The same procedure used to synthesise ethyl acetate (101) was employed to prepare ethyl [1,2-$^{13}C_2$]acetate (105), starting with sodium [1,2-$^{13}C_2$]acetate (103) (1.00 g, 11.90 mmol), giving ethyl [1,2-$^{13}C_2$]acetate (105) (0.61 g, 57%) as a colourless liquid. υ_{max} 1742 cm^{-1}; δ_H 1.26 (3H, t, *J* 7.0, $C\underline{H}_3CH_2$), 2.05 (3H, dd, *J* 130, 7.2, $^{13}C\underline{H}_3$), 4.18 (2H, dq, *J* 7.0, 3.0, $CH_3C\underline{H}_2O$); δ_C 21.05 (d, $^{13}\underline{C}H_3{}^{13}CO_2Et$), 171.18 (d, $^{13}CH_3{}^{13}\underline{C}O_2Et$); *m/z* 90 ($M^+$, 28%), 89 (7), 88 (1), 75 (29), 74 (12), 73 (7), 72 (55), 63 (100), 62 (25), and 61 (3); ^{13}C Incorporation, 85.1% with 2 x ^{13}C, 13.1% with 1 x ^{13}C, 1.8% with 0 x ^{13}C.

Diethyl [1,2-$^{13}C_2$]malonate (106)[77]

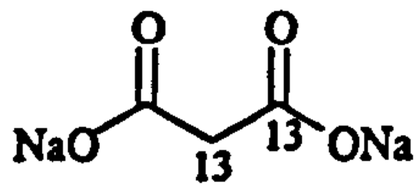


(106)

The same procedure used to synthesise diethyl malonate (102) was employed to prepare

diethyl [1,2-$^{13}C_2$]malonate (106), starting with hexamethyldisilazide (1.42 g, 8.80 mmol), n-butyl lithium (2.5M in hexanes, 3.5 cm^3, 8.75 mmol), ethyl [1,2-$^{13}C_2$]acetate (105) (0.50 g, 5.56 mmol), and ethyl chloroformate (0.96 g, 8.85 mmol). This method yielded diethyl [1,2-$^{13}C_2$]malonate (106) (0.41 g, 46%) as a colourless liquid. lit.[77] bp. 66 °C at 6 mmHg; υ_{max} 1759 cm^{-1}; δ_H 1.29 (6H, t, *J* 7, $C\underline{H}_3CH_2O$), 3.35 (2H, dd, *J* 132, 7.4, $^{13}C\underline{H}_2$), 4.18 (4H, m, $^{13}C\underline{H}_2$); δ_C 41.69 (d, $^{13}\underline{C}H_2$), 166.62 (d, $^{13}CH_2{}^{13}\underline{C}O_2Et$); *m/z* 162 ($M^+$, 4%), 161 (3), 160 (3), 117 (100), 116 (21), 115 (2.1), 91 (17), 90 (19), and 89 (37); ^{13}C Incorporation, 86.0% with 2 x ^{13}C, 12.3% with 1 x ^{13}C, 1.7% with 0 x ^{13}C.

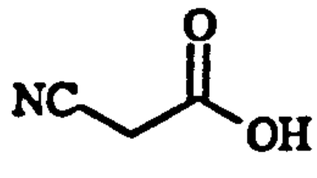
Disodium [1,2-$^{13}C_2$]malonate (104)[77]



(104)

The same procedure used to synthesise disodium malonate (97) was employed to synthesise disodium [1,2-$^{13}C_2$]malonate (104), starting with diethyl [1,2-$^{13}C_2$]malonate (106) (0.37 g, 2.28 mmol) was added sodium hydroxide solution (2M, 2.28 cm^3, 4.56 mmol). This method yielded disodium [1,2-$^{13}C_2$]malonate (104) (0.30 g, 88%) as a white crystalline solid. mp. >270 °C; υ_{max} (nujol) 1580 cm^{-1}; δ_H (^2H_2O) 3.15 (2H, dd, *J* 130, 6.4, $^{13}C\underline{H}_2$); δ_C (^2H_2O) 47.43 (d, $^{13}\underline{C}H_2$), 177.31 (d, $^{13}CH_2{}^{13}\underline{C}O_2Na$).

Cyanoacetic acid (108)[93]



(108)

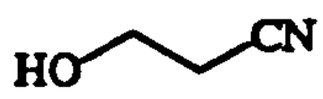
Bromoacetic acid (107) (2.42 g, 17.4 mmol) in distilled water (20 cm^3) was heated to 50 °C. The solution was then neutralised to the sodium salt, using sodium hydrogen carbonate (1 g, 11.9 mmol), and cooled to 0 °C. Sodium cyanide (0.93 g, 19.0 mmol) in distilled water (10 cm^3) was added, and the solution was left to warm to room temperature overnight. The solution was acidified to pH 1.0 using hydrochloric acid (2M), saturated with sodium chloride, and then extracted with ethyl acetate (4 x 75 cm^3). The combined organic extracts were then dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield cyanoacetic acid (108) (1.21 g, 82%) as a white crystalline solid. mp. 65-66 °C, (lit.,[136] 66-68 °C); υ_{max} (nujol) 2267, 1708 cm^{-1}; δ_H (d_6-acetone)

3.56 (2H, s, $C\underline{H}_2CN$), 6.15 (1H, br s, $CO_2\underline{H}$); δ_C (d_6-acetone) 24.25 ($\underline{C}H_2CO_2H$), 114.76 ($\underline{C}N$), 165.21 ($\underline{C}O_2H$); *m/z* 85 (M^+, 5%), 68 (15), 59 (73), 44 (100), and 41 (75).

Attempted reduction of cyanoacetic acid (108) to 3-hydroxypropionitrile, via a mixed anhydride

Oxalyl chloride (0.5 cm^3, 5.73 mmol) was added to a solution of DMF (0.2 cm^3, 2.57 mmol) in dichloromethane (3 cm^3) at 0 °C under a nitrogen atmosphere. The resulting solution was stirred for 1 hour, until a white precipitate formed. The solvent was then removed *in vacuo*. To the white residue was added dry acetonitrile (3 cm^3), THF (10 cm^3), and then cyanoacetic acid (108) (0.17 g, 2.00 mmol) at -30 °C, and the resulting solution was stirred for 1 h. Sodium borohydride (0.15 g, 3.95 mmol) was added at -30 °C. The solution was warmed to room temperature over 2 h., quenched with HCl (2M, 50 cm^3), washed with saturated sodium hydrogen carbonate (50 cm^3), and then extracted with diethyl ether (3 x 100 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield a pale yellow oil. The ^1H nmr spectrum indicated a complex mixture of products, and indicated no presence of the required product.

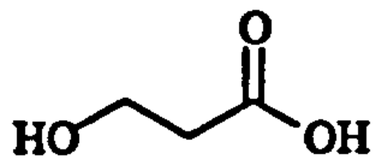
3-Hydroxypropionitrile (109)[95]



(109)

To 2-bromoethanol (110) (0.95 g, 7.60 mmol) in methanol (5 cm^3) was added potassium cyanide (0.35 g, 5.38 mmol) in water (10 cm^3). The mixture was heated at 48 °C for 9 hours. After this time, it was cooled in an ice-bath and extracted with diethyl ether (5 x 20 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by Kugelrohr distillation (125 °C, 1 mmHg) yielded 3-hydroxypropionitrile (109) (0.31 g, 81%) as a colourless oil. lit.,[95] bp. 107-109 °C at 12 mmHg; υ_{max} 3408, 2252 cm^{-1}; δ_H 2.62 (2H, t, *J* 6.4, $C\underline{H}_2CN$), 3.32 (1H, br s, $O\underline{H}$), 3.86 (2H, t, *J* 6.4 , $C\underline{H}_2OH$); δ_C 21.52 ($\underline{C}H_2CN$), 57.65 ($\underline{C}H_2OH$), 118.48 ($\underline{C}N$); *m/z* (CI) 71 (M^+, 3.9%), 70 (M^+-1, 6.5), and 54 (100).

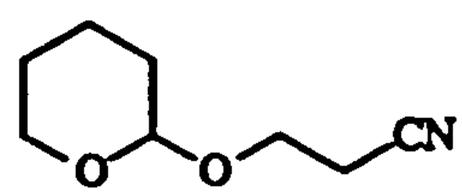
3-Hydroxypropionic acid (98)[95,98]



(98)

To 3-hydroxypropionitrile (109) (0.06 g, 0.85 mmol) was added sodium hydroxide solution (8mM, 100 cm^3), and the reaction mixture was stirred at room temperature for 12 hours. This was then acidified with sulphuric acid (4mM, 100 cm^3), stirred for 30 minutes, and then extracted with diethyl ether (5 x 50 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield 3-hydroxypropionic acid (98) (0.01 g, 13%) as a colourless oil. δ_H 2.62 (2H, m, $C\underline{H}_2CO_2H$), 4.84 (2H, br s, O$\underline{H}$ and $CO_2\underline{H}$); *m/z* (CI) 91 (MH^+, 6%), 90 (1), 89 (7), 73 (37), 72 (26), 71 (11), 56 (13), and 55 (100).

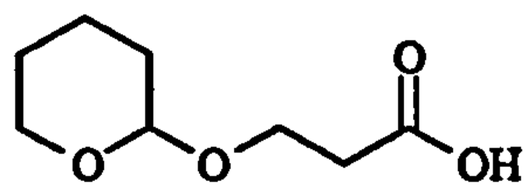
3-(1'-Tetrahydropyranyl-2-yl)oxypropionitrile (111)



(111)

Dihydropyran (2.41 g, 28.6 mmol) in dry dichloromethane (5 cm^3) was added dropwise to a solution of 3-hydroxypropionitrile (109) (2.01 g, 28.3 mmol) in dry dichloromethane (5 cm^3). A catalytic amount of pTSA was added, and the solution was stirred at room temperature overnight. The mixture was washed with saturated sodium hydrogen carbonate solution (50 cm^3), and then extracted with dichloromethane (4 x 50 cm^3). The combined organic extracts were dried with sodium sulphate, filtered and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 10% ethyl acetate/ petroleum ether 40-60 °C) yielded **3-(1'-tetrahydropyranyl-2-yl)oxypropionitrile (111)** (3.14 g, 72%) as a colourless oil. υ_{max} 2252 cm^{-1}; δ_H 1.54-1.85 (6H, m, 3 x $C\underline{H}_2$), 2.65 (2H, t, *J* 6.0, $C\underline{H}_2CN$), 3.52-3.69 (2H, m, $OCHOC\underline{H}_2CH_2CH_2$), 3.82-3.97 (2H, m, $OC\underline{H}_2CH_2CN$), 4.67 (1H, m, OC$\underline{H}$O); *m/z* (CI) 156 (MH^+, 18%), 155 (M^+, 6), 85 (91), 72 (24), 71 (7), and 57 (11). Found MH^+, 156.1037 $C_8H_{13}NO_2$ requires MH^+, 156.1025.

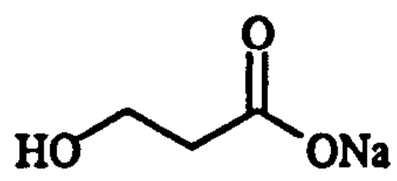
3-(1'-Tetrahydropyranyl-2-yl)oxypropionic acid (112)



(112)

To 3-(1'-tetrahydropyranyl-2-yl)oxypropionitrile (111) (0.13 g, 0.84 mmol) was added sodium hydroxide solution (8mM, 100 cm^3), and the reaction mixture was stirred at room temperature for 12 hours. The mixture was then acidified with sulphuric acid (4mM, 100 cm^3), stirred for 0.5 h., and then extracted with diethyl ether (3 x 50 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield a yellow oil. The ^1H nmr spectrum indicated a complex mixture, no signals of which corresponded to the required product.

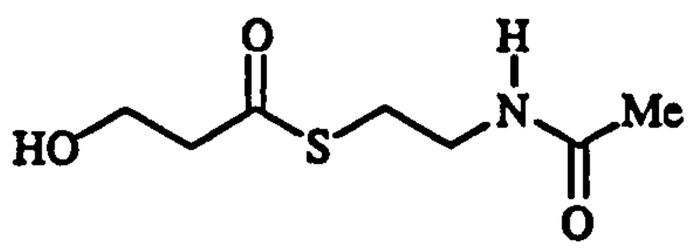
Sodium 3-hydroxypropionate (113)[99]



(113)

To 3-hydroxypropionitrile (109) (2.00 g, 28.2 mmol) was added 2M sodium hydroxide solution (14.1 cm^3, 28.2 mmol). This was heated to reflux overnight at 75 °C. The solution was cooled in ice, water (20 cm^3) was added, and then the solution was extracted with ethyl acetate (20 cm^3). The organic layer was then backwashed with water (2 x 20 cm^3). The combined aqueous layers were extracted, and the water was removed *in vacuo*. The residue was recrystallised from ethanol, and then freeze-dried to leave sodium 3-hydroxypropionate (113) (3.00 g, 95%) as a white crystalline solid. mp. 140-142 °C (lit.,[137] 143 °C); υ_{max} (nujol) 3367, 1571 cm^{-1}; δ_H (^2H_2O) 2.40 (2H, t, *J* 6, $C\underline{H}_2CO_2Na$), 3.78 (2H, t, *J* 6, $C\underline{H}_2OH$); δ_C (^2H_2O) 39.97 ($\underline{C}H_2CO_2Na$), 58.83 ($\underline{C}H_2OH$), 180.42 ($\underline{C}O_2Na$).

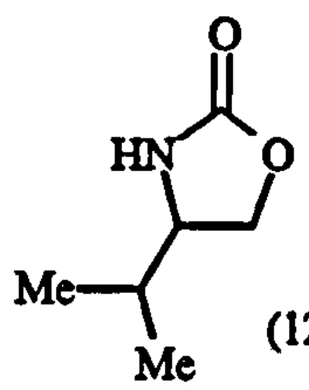
S-2-(Acetylamino)ethyl 3-hydroxypropanethioate (99) (N-acetylcysteamine thioester of 3-hydroxypropionic acid)



(99)

To freshly prepared N-acetylcysteamine (73) (0.27 g, 2.27 mmol) was added dichloromethane (3 cm^3) with stirring at 0 °C under a nitrogen atmosphere. Dicyclohexylcarbodiimide (83) (0.38 g, 1.84 mmol) in dichloromethane (4 cm^3) was added, followed by 4-dimethylaminopyridine (84) (0.01 g, 0.08 mmol), in dichloromethane (1 cm^3). 3-Hydroxypropionic acid (98) (0.16 g, 1.78 mmol) in dichloromethane (5 cm^3) was then added dropwise. An immediate white precipitate of dicyclohexylurea formed. The solution was left at 0 °C for 3 hours and was then allowed to warm to room temperature overnight. The resulting solution was filtered through Celite to remove the precipitate. To the filtrate was added saturated ammonium chloride (25 cm^3), and was extracted with dichloromethane (2 x 20 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 95% ethyl acetate, 5% methanol) yielded the **S-2-(acetylamino)ethyl 3-hydroxypropanethioate (99)** (0.11 g, 32%) as a colourless oil. υ_{max} 3386, 1701, 1685 cm^{-1}; δ_H 2.82 (2H, m, $C\underline{H}_2COS$), 3.08 (2H, t, *J* 6.5, $C\underline{H}_2S$), 3.45 (2H, q, *J* 6.5, $C\underline{H}_2NH$), 3.92 (2H, t, $C\underline{H}_2OH$), 6.20 (1H, br s, N<u>H</u>); δ_C 23.12 ($\underline{C}H_2NH$), 28.81 ($\underline{C}H_2S$), 39.14 ($\underline{C}H_3CON$), 46.62 ($\underline{C}H_2COS$), 61.42 ($\underline{C}H_2OH$), 171.06 (<u>C</u>ON), 191.43 (<u>C</u>OS); *m/z* (CI) 192 (MH^+, 12%), 174 (100), 132 (60), 120 (55), 118 (59), 86 (76), 73 (22), 55 (30). Found (M-OH)$^+$, 174.0591 $C_8H_{13}NO_2$ requires (M-OH)$^+$, 174.0589.

4-(1-Methylethyl)-2-oxazolidonone (122)[100]

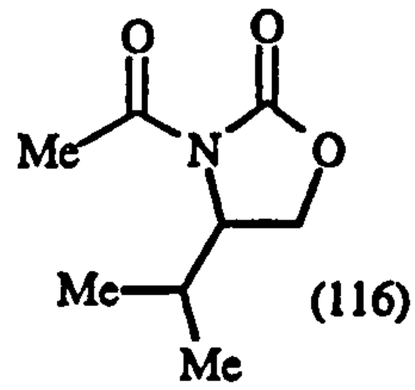


(122)

A round bottomed flask (250 cm^3), fitted with a vigreux column (20cm), and equipped with a distillation set-up, was charged with (*RS*)-amino-3-methylbutan-1-ol (121) (10.00 g, 98.0 mmol), potassium carbonate (0.14 g, 1.0 mmol), and diethyl carbonate (23.8 cm^3, 196.0 mmol). The solution was heated and stirred to 125 °C, until approximately 12 cm^3 of ethanol had been collected. To the remaining solution was added diethyl ether (300 cm^3). The reaction was then filtered through Celite under vacuum, in order to remove potassium carbonate. Diethyl ether was removed *in vacuo*, to yield a white solid, which was recrystallised from ethyl acetate/petroleum 40-60 °C to yield a white crystalline solid. Concentration of the mother liquors gave a second crop of white crystals, so yielding 4-(1-methylethyl)-2-oxazolidonone (122) (8.39 g, 66%) as a white crystalline solid. mp. 69-70 °C (lit.,[100] 71-72 °C); υ_{max} (nujol) 1743 cm^{-1}; δ_H 0.90 (3H, d, *J* 6.5,

$C\underline{H}_3CH$), 0.96 (3H, d, *J* 6.5, $C\underline{H}_3CH$), 1.73 (1H, m, $C\underline{H}(CH_3)_2$), 3.61 (1H, q, *J* 6.5, $OCH_2C\underline{H}NH$), 4.10 (1H, dd, *J* 8.5, 6.5, $OC\underline{H}HCHNH$), 4.44 (1H, t, *J* 8.5, $OC\underline{H}HCHNH$), 6.63 (1H, br s, $N\underline{H}$); δ_C 17.60 ($\underline{C}H_3CH$), 17.91 ($\underline{C}H_3CH$), 32.64 ($\underline{C}H(CH_3)_2$), 58.38 ($CH_2\underline{C}HNH$), 68.29 ($\underline{C}H_2CHNH$), 160.42 ($\underline{C}$=O); *m/z* 129 (M^+, 5%), 114 (2), 100 (11), 87 (13), 86 (100), 85 (45), 58 (13), and 56 (10).

3-Acetyl-4-(1-methylethyl)-2-oxazolidonone (116)[100]



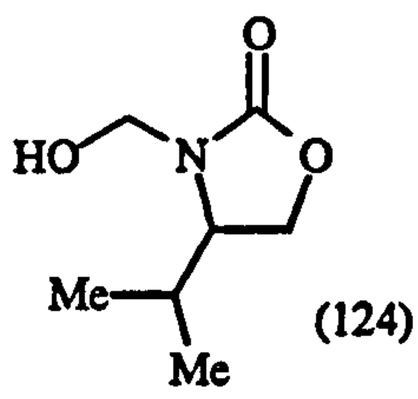
(116)

Sodium acetate (100) (1.00 g, 12.2 mmol) was suspended in THF (50 cm^3) under a nitrogen atmosphere. Pivaloyl chloride (1.7 cm^3, 13.4 mmol) was then added as a concentrated solution, and the reaction was stirred at room temperature for 48 hours. After this time a white precipitate, of sodium chloride, in solution was observed. The mixed anhydride (123) was used immediately in the acylation reaction, without further analysis. To THF (40 cm^3) under a nitrogen atmosphere at -78 °C was added (4*S*)-4-(1-methylethyl)-2-oxazolidonone (122) (1.72 g, 13.3 mmol). Butyl lithium (1.6M, 9.5 cm^3, 15.2 mmol) was added, and stirred for 20 minutes at -78 °C. A white precipitate immediately formed. The anion was added to the mixed anhydride under a nitrogen atmosphere at -78 °C. No change was observed on addition. This solution was stirred at -78 °C for 6 hours, then washed with saturated ammonium chloride (50 cm^3), and then extracted with ethyl acetate (4 x 75 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered, and concentrated *in vacuo*. Purification by flash column chromatography, eluting with 5% ethyl acetate/petroleum ether 40-60 °C yielded 3-tert-butyl-4-(1-methylethyl)-2-oxazolidonone oxazolidonone as a pale yellow oil.[82] υ_{max} 1788, 1688 cm^{-1}; δ_H 0.86 (3H, d, *J* 6.5, $C\underline{H}_3CH$), 0.90 (3H, d, *J* 6.5, $C\underline{H}_3CH$), 1.39 (9H, s, $C(C\underline{H}_3)_3$), 2.23 (1H, m, $C\underline{H}(CH_3)_2$), 4.18 (1H, m, $OC\underline{H}HCHN$), 4.27 (1H, m, $OC\underline{H}HCHN$), 4.52 (1H, m, $OCH_2C\underline{H}N$); *m/z* 213 (M^+, 1%), 158 (50), 142 (10), 129 (12), 86 (62), 85 (83), 83 (100), and 57 (88).

Eluting with 10% ethyl acetate/petroleum ether 40-60 °C yielded 3-acetyl-4-(1-methylethyl)-2-oxazolidonone (116) (0.88 g, 84%) as a pale yellow oil. The yield is based on recovered starting material. υ_{max} 1784, 1702 cm^{-1}; δ_H 0.88 (3H, d, *J* 6.5, $C\underline{H}_3CH$), 0.92 (3H, d, *J* 6.5, $C\underline{H}_3CH$), 2.40 (1H, m, $C\underline{H}(CH_3)_2$), 2.54 (3H, s, $C\underline{H}_3CO$), 4.24 (2H, m, $OC\underline{H}HCHN$ and $OC\underline{H}HCHN$), 4.43 (1H, m, $OCH_2C\underline{H}N$); δ_C 17.97 ($\underline{C}H_3CH$), 23.78 ($\underline{C}H_3CH$), 27.02 ($\underline{C}H(CH_3)_2$), 28.35 ($\underline{C}H_3CO$), 58.35 ($\underline{C}HN$), 63.31 ($\underline{C}H_2O$), 154.29 ($\underline{C}$=O), 170.33 ($CH_3\underline{C}ON$); *m/z* 171 (M^+, 31%), 158 (10), 129

(7), 128 (67), 86 (100), 71 (7), 68 (14), and 57 (32).
Further eluting with 50% ethyl acetate/petroleum ether 40-60 °C yielded the starting material, 4-(1-methylethyl)-2-oxazolidonone (122) (0.93 g), as a white crystalline solid. Spectral data as before.

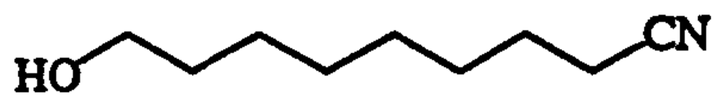
Reaction of (116) with sodium hexamethyldisilylazide and paraformaldehyde



(124)

A solution of 3-acetyl-4-(1-methylethyl)-2-oxazolidonone (116) (0.21 g, 1.21 mmol) in THF (2 cm^3) at -78 °C under a nitrogen atmosphere was added over 5 minutes to sodium hexamethyldisilazide (1M in THF, 1.5 cm^3, 1.50 mmol), and the solution was stirred for 0.5 h. Hexamethylphosphoramide (0.43 g, 0.42 cm^3, 2.40 mmol) was added, immediately followed by formaldehyde (large excess). (This was generated by the depolymerisation of paraformaldehyde by heating paraformaldehyde in an oil bath, and by passing the formaldehyde gas over a steady stream of nitrogen). The reaction mixture was stirred for 1 hour at -78 °C, and then quenched by addition of saturated ammonium chloride (50 cm^3). The mixture was then acidified to pH 2.0 with 2M hydrochloric acid, and extracted with ethyl acetate (3 x 40 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 30% ethyl acetate/petroleum ether 40-60 °C) yielded **3-hydroxymethyl-4-(1-methylethyl)-2-oxazolidonone (124)** (0.16 g, 83%) as a colourless oil. δ_H 0.91 (3H, d, *J* 5.2, C$\underline{H}_3$CH), 0.94 (3H, d, *J* 5.2, C$\underline{H}_3$CH), 2.15 (1H, m, C$\underline{H}$(CH_3)$_2$), 3.29 (1H, br s, O$\underline{H}$), 4.01 (1H, m, OC$\underline{H}$HCHN), 4.12 (1H, m, OC$\underline{H}$HCHN), 4.28 (1H, m, $OCH_2C\underline{H}N$), 4.68 (1H, d, *J* 11.6, HOC$\underline{H}$HN), 4.95 (1H, d, *J* 11.6, HOC$\underline{H}$HN); *m/z* (CI) 159 (M^+, 1%), 158 (M^+-1, 2), 142 (M^+-OH, 36), 130 (100), 129 (3), 86 (13), and 84 (11). Found MH^+, 160.0970 $C_7H_{14}NO_4$ requires MH^+, 160.0973.

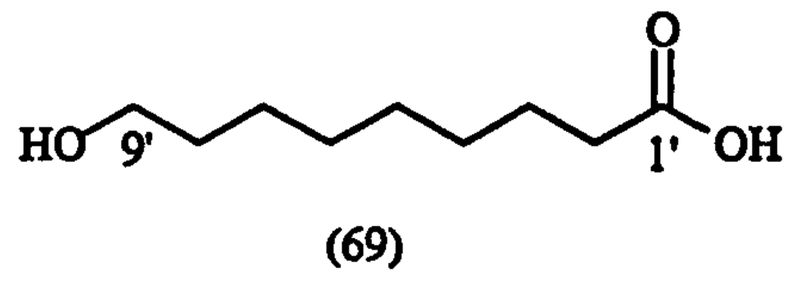
9-Hydroxynonanitrile (213)



(213)

To a stirred solution of 8-bromo-1-octanol (3.11 g, 14.9 mmol) in distilled methanol (15 cm^3) was added sodium cyanide (0.73 g, 14.9 mmol) in distilled water (30 cm^3). The reaction mixture was then heated to reflux for 16 hours. This solution was cooled in an ice-bath to 0 °C, and then extracted with ethyl acetate (3 x 75 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered, and concentrated *in vacuo*. Purification by Kugelrohr distillation (125 °C, 0.5 mmHg) yielded **9-hydroxynonanitrile (213)** (1.75 g, 76%) as a colourless oil. υ_{max} 3330, 2245 cm^{-1}; δ_H 1.25-1.72 (12H, m, 6 x C$\underline{H}_2$), 2.34 (2H, t, *J* 7.2, C$\underline{H}_2$CN), 3.64 (2H, t, *J* 6.4, C$\underline{H}_2$OH); *m/z* 155 (M^+, 1%), 154 (6), 138 (13), 110 (32), 97 (92), 82 (67), 69 (69), and 55 (100). Found MH^+, 156.1391 $C_9H_{18}NO$ requires MH^+, 156.1388.

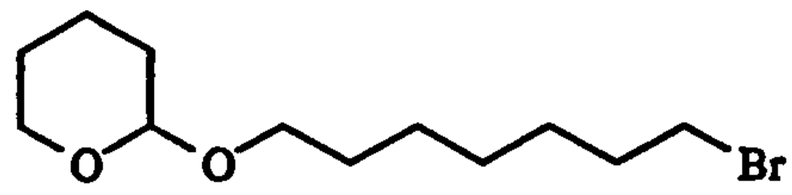
9-Hydroxynonanoic acid (69)



(69)

To 9-hydroxynonanitrile (213) (0.3 g, 1.94 mmol) was added 2M sodium hydroxide solution (1 cm^3, 2.00 mmol), and the resulting mixture was heated to reflux for 72 hours. The aqueous layer was extracted with ethyl acetate (20 cm^3). The aqueous extract was acidified to pH 1.0 using hydrochloric acid (2M), stirred at room temperature for 15 minutes, and then extracted with ethyl acetate (5 x 20 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield **9-hydroxynonanoic acid (69)** (0.33 g, 98%) as a white crystalline solid. mp. 43-44 °C; υ_{max} (nujol) 3367, 1734 cm^{-1}; δ_H (d_6-acetone) 1.31-1.71 (12H, m, 6 x C$\underline{H}_2$), 2.28 (2H, t, *J* 7.4, C$\underline{H}_2CO_2H$), 2.88 (1H, br s, O$\underline{H}$), 3.52 (2H, t, *J* 6.4, C$\underline{H}_2$OH), 10.46 (1H, br s, $CO_2\underline{H}$); δ_C (d_6-acetone) 25.01, 25.99, 28.63, 28.91, 29.20, 29.61 (C-3', C-4', C-5', C-6', C-7', C-8'), 33.55 ($\underline{C}H_2CO_2H$), 61.81 ($\underline{C}H_2OH$), 174.06 ($\underline{C}O_2H$); *m/z* (CI) 175 (MH^+, 13%), 174 (8), 157 (43), 139 (100), 121 (18), 97 (23), 69 (12), and 55 (6). Found $(M\text{-}OH)^+$, 157.1231 $C_9H_{17}O_2$ requires $(M\text{-}OH)^+$, 157.1229.

7-Bromo-1-(1'-tetrahydropyran-2-yl)oxyheptane (136)[106]



(136)

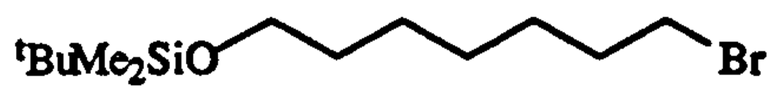
(a) Using pTSA and dihydropyran

To 7-bromo-1-heptanol (135) (3.26 g, 16.7 mmol) was added freshly distilled diethyl ether (30 cm^3). Dihydropyran (1.55 g, 18.4 mmol) in diethyl ether (5 cm^3) was added dropwise. pTSA (0.07 g, 0.37 mmol) was added, and the solution was stirred at room temperature for 24 hours. The mixture was then extracted with diethyl ether (2 x 50 cm^3). The combined organic extracts were washed with 2M sodium hydroxide solution (50 cm^3), water (50 cm^3) and brine (50 cm^3). They were then dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by Kugelrohr distillation (235 °C, 3 mmHg) yielded 7-bromo-1-(1'-tetrahydropyran-2-yl)oxyheptane (136) (4.30 g, 92%) as a colourless oil. lit. bp.,[106] 125 °C at 1 mmHg; υ_{max} 726 cm^{-1}; δ_H 1.35-1.92 (16H, m, 8 x C$\underline{H}_2$), 3.41 (2H, t, *J* 6.8, C$\underline{H}_2$Br), 3.46-3.92 (4H, m, 2 x C$\underline{H}_2$O), 4.57 (1H, t, *J* 3.5, OC$\underline{H}$O); δ_C 19.65, 25.43, 26.02, 28.05, 29.59, 30.64, 32.57, 34.08, 62.32 ($\underline{C}H_2$O), 67.48 ($\underline{C}H_2$O), 98.82 (O$\underline{C}$HO); *m/z* 280 (M^+, 1%) & 278 (M^+, 1), 279 ($(M-1)^+$, 5) & 277 ($(M-1)^+$, 5), 225 (1) & 223 (1), 207 (1) & 205 (1), 179 (3) & 177 (3), 150 (2) & 148 (2), 97 (37), 85 (100), 56 (41), and 55 (49).

(b) Using conc. HCl and dihydropyran

To a stirred solution of 7-bromo-1-heptanol (135) (1.08 g, 5.54 mmol) in concentrated HCl (0.3 cm^3) was added dihydropyran (0.78 g, 9.27 mmol). The reaction mixture was heated to reflux for 2 days, and then cooled in ice. This was washed with benzene (2 x 30 cm^3), sodium hydrogen carbonate (3 x 20 cm^3), brine (2 x 20 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 15% ethyl acetate/petroleum ether 40-60 °C) yielded 7-bromo-1-(1'-tetrahydropyran-2-yl)oxyheptane (136) (0.27 g, 18%) as a colourless oil. Spectral data as before.

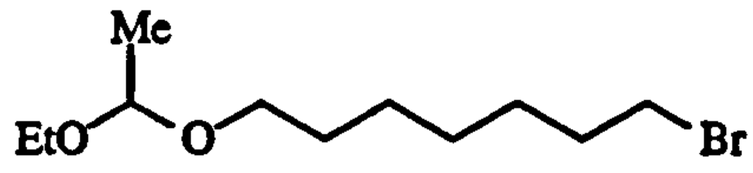
7-Bromo-1-(1'-tert-butyldimethylsilyl)oxyheptane (138)



(138)

To 7-bromo-1-heptanol (135) (10.00 g, 51.3 mmol) was added dimethylformamide (20 cm^3). TBDMS chloride (8.30 g, 55.1 mmol) and imidazole (6.80 g, 100.0 mmol) were then added. This solution was then stirred at room temperature, under a nitrogen atmosphere, for 20 hours. Distilled water (50 cm^3) and diethyl ether (50 cm^3) were added, followed by extraction with diethyl ether (3 x 50 cm^3). The combined organic extracts were washed with hydrochloric acid solution (2M, 50 cm^3) and brine (50 cm^3). They were then dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield **7-bromo-1-(1'-tert-butyldimethylsilyl)oxyheptane (138)** (15.2 g, 96%) as a colourless oil. δ_H 0 (6H, s, $(C\underline{H}_3)_2Si$), 0.90 (9H, s, $(C\underline{H}_3)_3C$), 1.32-1.56 (8H, m, 4 x $C\underline{H}_2$), 1.71-1.90 (2H, m, 1 x $C\underline{H}_2$), 3.35 (2H, t, *J* 7.0, $C\underline{H}_2Br$), 3.55 (2H, t, *J* 6.6, $C\underline{H}_2O$); δ_C 0, 18.38, 25.65, 26.00, 28.16, 28.57, 32.72, 34.00, 63.18; *m/z* (CI) 309 ($(M-1)^+$, 1%) & 307 ($(M-1)^+$, 1), 253 (1) & 251 (1), 179 (4) & 177 (4), 115 (14), 97 (100), 75 (35), and 55 (96).

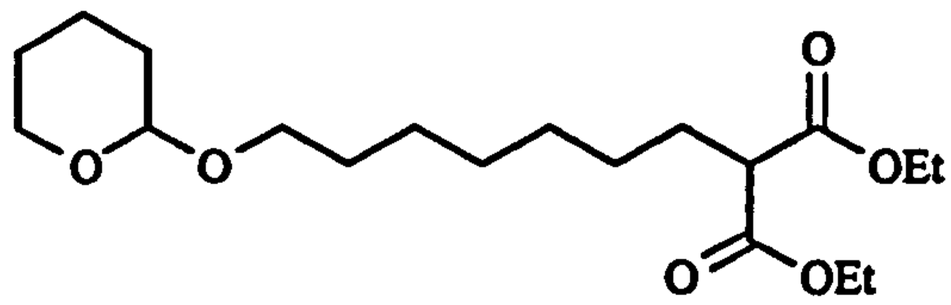
7-Bromo-1-(1'-ethoxy)oxyheptane (139)



(139)

To 7-bromo-1-heptanol (135) (2.51 g, 12.9 mmol) was added ethoxyethene (15 cm^3, 157 mmol), together with a catalytic amount of trifluoroacetic acid. This solution was stirred at room temperature, under a nitrogen atmosphere, for 24 hours. A dark brown solution resulted, after which time the solvent was removed *in vacuo*. Purification by Kugelrohr distillation (125 °C, 1 mmHg) yielded **7-bromo-1-(1'-ethoxy)oxyheptane (139)** (2.97 g, 87%) as a colourless oil. υ_{max} 726 cm^{-1}; δ_H 1.21 (3H, t, *J* 7.5, $C\underline{H}_3CH_2$), 1.28 (3H, d, *J* 6.0, $OCH(C\underline{H}_3)O$), 1.30-1.60 (8H, m, 4 x $C\underline{H}_2$), 1.80-1.91 (2H, m, $C\underline{H}_2CH_2OH$), 3.41 (2H, t, *J* 7.0, $C\underline{H}_2Br$), 3.40-3.80 (4H, m, 2 x $C\underline{H}_2O$), 4.65 (1H, q, *J* 3.5, $OC\underline{H}O$); δ_C 15.31, 20.00, 26.14, 28.11, 28.56, 29.79, 32.59, 34.02, 60.61 ($CH_3\underline{C}H_2O$), 65.11 ($O\underline{C}H_2CH_2$), 99.49 ($O\underline{C}HO$); *m/z* 269 (MH^+, 1%) & 267 (MH^+, 2), 253 (5) & 251 (6), 223 (17) & 221 (17), 179 (14) & 177 (14), 150 (42) & 148 (43), 137 (7) & 135 (7), 97 (84), 73 (89), 69 (72), and 55 (100).

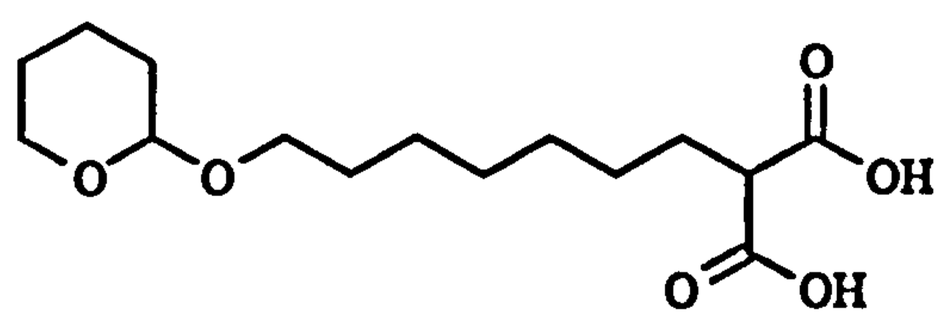
Diethyl 9-(1'-tetrahydropyran-2-yl)oxynon-1-ylmalonate (144)



(144)

A 50 cm^3 round bottomed flask, equipped with a reflux condenser, was charged with diethyl malonate (102) (1.50 g, 9.38 mmol), 7-bromo-1-(1'-tetrahydropyran-2-yl)oxyheptane (136) (2.87 g, 10.29 mmol), and acetonitrile (6 cm^3), and was stirred under a nitrogen atmosphere. To this solution was added 18-Crown-6 (0.09 g, 0.36 mmol), and potassium carbonate (1.49 g, 10.8 mmol), and this was heated to reflux for 24 hours. This solution was cooled in an ice-bath, and extracted with dichloromethane (3 x 50 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered, and concentrated *in vacuo*. Purification by Kugelrohr distillation (225 °C, 2 mmHg) yielded **diethyl 9-(1'-tetrahydropyran-2-yl)oxynon-1-ylmalonate (144)** (3.29 g, 98%) as a colourless oil. υ_{max} 1733 cm^{-1}; δ_H 1.27 (6H, t, *J* 7.0, 2 x $C\underline{H}_3CH_2O$), 1.51-1.88 (18H, m, 9 x $C\underline{H}_2$), 3.31 (1H, t, *J* 7.0, $C\underline{H}(CO_2Et)_2$), 3.35-3.91 (4H, m, 2 x $C\underline{H}_2O$), 4.17 (4H, q, J 7.0, 2 x $CH_3C\underline{H}_2O$), 4.57 (1H, t, *J* 3.5, $OC\underline{H}O$); δ_C 14.05 ($\underline{C}H_3CH_2O$), 19.66, 25.69, 26.08, 27.22, 28.52, 28.68, 29.13, 29.63, 30.71, 51.87 ($\underline{C}H(CO_2Et)_2$), 60.87 ($CH_3\underline{C}H_2O$), 62.30 ($O\underline{C}H_2CH_2$), 67.54 ($O\underline{C}H_2$), 98.81 ($O\underline{C}HO$), 169.54 (2 x $\underline{C}O_2Et$); *m/z* (CI) 389 (100), 359 (MH^+, 5%), 358 (M^+, 1), 276 (13), 275 (77), and 85 (66) . Found M^+, 359.2365 $C_{19}H_{34}O_6$ requires M^+, 359.2374.

9-(1'-Tetrahydropyran-2-yl)oxynon-1-ylmalonic acid (145)

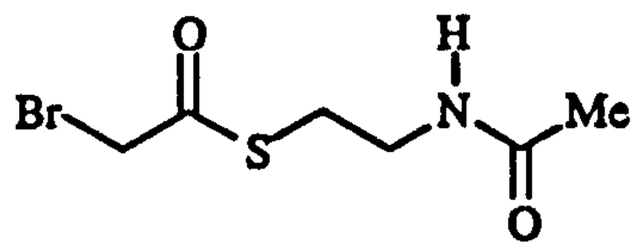


(145)

To diethyl 9-(1'-tetrahydropyran-2-yl)oxynonylmalonate (144) (1.00 g, 2.79 mmol) was added sodium hydroxide solution (2M, 2.79 cm^3, 5.58 mmol), together with the phase transfer catalyst, tetrabutyl ammonium bromide (10mg). The mixture was stirred at room temperature for 48 hours. This solution was acidified to pH 1.0 with hydrochloric acid (2M), and extracted with ethyl acetate (4 x 20 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered, and concentrated *in vacuo* to yield **9-(1'-tetrahydropyran-2-yl)oxynon-1-ylmalonic acid (145)** (0.75 g, 89%) as a colourless oil. υ_{max} 3324, 1713 cm^{-1}; δ_H 1.20-1.94 (18H, m, 9 x $C\underline{H}_2$), 3.41 (1H, t, *J*

7.0, C<u>H</u>(CO_2H)$_2$), 3.48-3.93 (4H, m, 2 x C<u>H$_2$</u>O), 4.60 (1H, t, *J* 3.5, OC<u>H</u>O), 9.45 (2H, br s, CH(CO_2<u>H</u>)$_2$); δ_C 19.60, 25.37, 25.57, 26.87, 27.62, 28.87, 29.07, 29.63, 30.71, 51.06 (<u>C</u>H(CO_2H)$_2$), 62.46 (O<u>C</u>H$_2$CH$_2$), 67.76 (O<u>C</u>H$_2$), 98.98 (O<u>C</u>HO), 173.64 (2 x <u>C</u>O$_2$H); *m/z* 302 (M^+, 1%), 256 (1), 173 (41), 101 (11), 97 (33), 85 (14), 84 (28), and 55 (100).

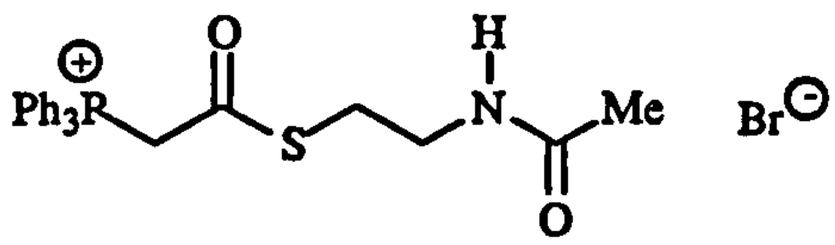
S-2-(Acetylamino)ethyl bromoethanethioate (152) (N-acetylcysteamine thioester of bromoacetic acid)



(152)

To freshly prepared N-acetylcysteamine (73) (3.58 g, 30.1 mmol) was added freshly distilled dichloromethane (10 cm^3). This was stirred at 0 °C under a nitrogen atmosphere. Dicyclohexylcarbodiimide (83) (4.99 g, 24.2 mmol) in distilled dichloromethane (10 cm^3) was added, followed by 4-dimethylaminopyridine (84) (0.16 g, 1.30 mmol), in distilled dichloromethane (3 cm^3). Bromoacetic acid (107) (3.18 g, 22.9 mmol) in distilled dichloromethane (10 cm^3) was then added dropwise. An immediate white precipitate of dicyclohexylurea formed. The solution was left at 0 °C for 3 hours and was then allowed to warm to room temperature overnight. The resulting solution was filtered through Celite to remove the precipitate. To the filtrate was added saturated ammonium chloride (50 cm^3). This was extracted with dichloromethane (2 x 20 cm^3), after which the organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Recrystallisation from ethyl acetate yielded the **S-2-(acetylamino)ethyl bromoethanethioate (152)** (4.67 g, 85%) as a white crystalline solid. mp. 95-97 °C; υ_{max} (nujol) 3260, 1665, 1628, 723 cm^{-1}; δ_H 1.99 (3H, s, C<u>H$_3$</u>CO), 3.11 (2H, t, *J* 6.5, C<u>H$_2$</u>S), 3.47 (2H, q, *J* 6.5, C<u>H$_2$</u>NH), 4.06 (2H, s, BrC<u>H$_2$</u>CO), 6.02 (1H, br s, N<u>H</u>); δ_C 23.16 (<u>C</u>H$_2$N), 29.70 (<u>C</u>H$_2$S), 33.32 (<u>C</u>H$_2$Br), 39.03 (<u>C</u>H$_3$CO), 170.40 (<u>C</u>ON), 193.07 (<u>C</u>OS); *m/z* (CI) 242 (MH^+, 4%) & 240 (MH^+, 4), 225 (7), 198 (4), 161 (5), 160 (13), 120 (95) & 118 (100). Found M^+, 239.9870 $C_6H_{10}NO_2SBr$ requires M^+, 239.9871.

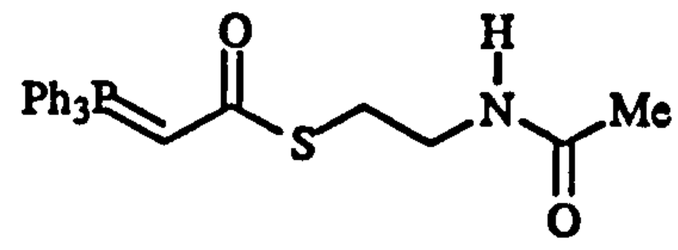
S-2-(Acetylamino)ethyl triphenylphosphonium ethanethioate bromide (153) (N-acetylcysteamine thioester of (triphenylphosphonium acetate) bromide)



(153)

The N-acetylcysteamine thioester (152) of bromoacetic acid (2.28 g, 9.50 mmol) was suspended in distilled toluene (40 cm^3). Triphenylphosphine (2.51 g, 9.58 mmol) was added, and the mixture was stirred for 4 days under a nitrogen atmosphere. The reaction mixture was filtered under vacuum Recystallisation from ethyl acetate, followed by the solid being dried over phosphorous pentoxide in a dessicator overnight, yielded the **S-2-(acetylamino)ethyl triphenylphosphonium ethanethioate bromide (153)** (3.42 g, 72%) as a pale yellow solid. mp. >250 °C; υ_{max} (nujol) 3285, 1731, 1665 cm^{-1}; δ_H 1.95 (3H, s $C\underline{H}_3CO$), 3.02 (2H, t, *J* 5.5, $C\underline{H}_2S$), 3.32 (2H, q, *J* 6.0, $C\underline{H}_2NH$), 5.81 (2H, d, *J* 12.5, $C\underline{H}_2P$), 7.50 -7.83 (15H, m, $(C_6\underline{H}_5)_3P$), 8.17 (1H, t, *J* 6.0, N$\underline{H}$); δ_C 22.97 ($\underline{C}H_2N$), 30.52 ($\underline{C}H_2S$), 37.95 ($\underline{C}H_3CO$), 117.19 ($\underline{C}H_2P$), 118.49 ($(\underline{C}\text{-}P)_3$), 130.11 & 130.30 (p-$(\underline{C}_6H_5)_3P$), 133.76 & 133.92 (m-$(\underline{C}_6H_5)_3P$), 135.07 & 135.21 (o-$(\underline{C}_6H_5)_3P$), 170.52 (CON), 181.66 (COS); *m/z* (FAB) 422 $((M\text{-}Br)^+$, 100%), 337 (27), 303 (72), 275 (34), 262 (6), 183 (16), 153 (7), and 133 (16).

S-2-(Acetylamino)ethyl triphenylphosphorane ethanethioate (151) (N-acetylcysteamine thioester of triphenylphosphorane acetate)

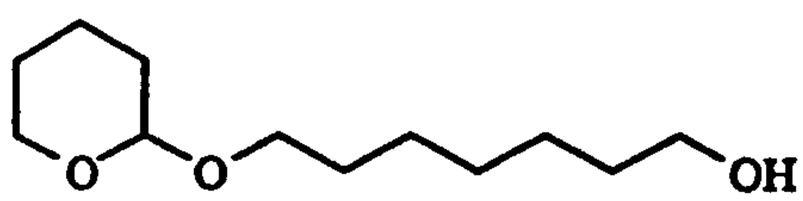


(151)

The N-acetylcysteamine thioester (153) of (triphenylphosphonium acetate) bromide (0.53 g, 1.1 mmol) was dissolved in water (30 cm^3). The solution was adjusted to pH 9.0 with 2M sodium hydroxide, and then extracted with dichloromethane (5 x 25 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield the **S-2-(acetylamino)ethyl triphenylphosphorane ethanethioate (151)** as a white crystalline solid (0.35 g, 79%). This was used without further purification. υ_{max} (nujol) 3270, 1731, 1625 cm^{-1}; δ_H 1.70 (3H, s, $C\underline{H}_3CON$), 2.99 (2H, t, *J* 6.0, $C\underline{H}_2S$), 3.37 (2H, q, *J* 6.0, $C\underline{H}_2N$), 3.85 (1H, br s, C$\underline{H}$P), 7.43-7.73 (15H, m, $(C_6\underline{H}_5)_3P$); δ_C 23.21 ($\underline{C}H_2N$), 27.62 ($\underline{C}H_2S$), 43.04 ($\underline{C}H_3CO$), 48.08 ($\underline{C}$HP), 126.78 & 128.97 (p-$(\underline{C}_6H_5)_3P$), 129.32 & 132.30 (m-$(\underline{C}_6H_5)_3P$), 133.04 &

133.12 (o-($\underline{C}_6H_5)_3P$), 170.52 (CON), 181.66 (COS), *m/z* 303 ((M-118)$^+$, 2%), 119 (20), 118 (12), 77 (15), 72 (17), 60 (81), 43 (76), 30 (100).

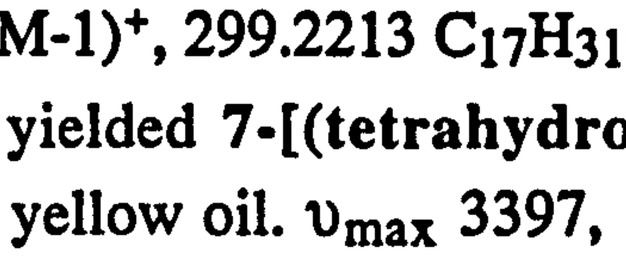
7-(1'-Tetrahydropyran-2-yl)oxyheptan-1-ol (156)



(156)

To a stirred solution of 1,7-heptanediol (155) (3.00 g, 22.73 mmol) in diethyl ether (20 cm^3) was added dihydropyran (1.92 g, 22.82 mmol) in diethyl ether (5 cm^3) and a catalytic amount of pTSA (0.1 g, 0.53 mmol). The mixture was stirred at room temperature for 18 hours, then quenched with water (100 cm^3), and was extracted with ethyl acetate (3 x 50 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*, and then purified by flash column chromatography (SiO_2, 35% ethyl acetate/ petroleum ether 40-60 °C) to yield two compounds. One band at R_f=0.78 yielded **1,7-[(tetrahydropyran-2-yl)oxy]heptane** (1.37 g, 20%) as a pale yellow oil. υ_{max} 1400-1000 cm^{-1}; δ_H 1.36-1.92 (22H, m, 11 x $C\underline{H}_2$), 3.33-3.91 (8H, m, 4 x $C\underline{H}_2O$), 4.58 (1H, t, *J* 3.5, OC$\underline{H}$O); δ_C 19.71, 25.48, 26.16, 29.32, 29.66, 30.79, 62.41 (O$\underline{C}H_2CH_2$), 67.72 (O$\underline{C}H_2$), 98.90 (O$\underline{C}$HO); *m/z* (CI) 299 ((M-1)$^+$, 1%), 215 (6), 199 (3), 185 (2), 133 (9), 86 (13), 85 (100), and 55 (22). Found (M-1)$^+$, 299.2213 $C_{17}H_{31}O_4$ requires (M-1)$^+$, 299.2222. The second band at R_f=0.55 yielded **7-[(tetrahydropyran-2-yl)oxy]heptan-1-ol (156)** (1.92 g, 39%) as a pale yellow oil. υ_{max} 3397, 1400-1000 cm^{-1}; δ_H 1.30-1.88 (16H, m, 8 x $C\underline{H}_2$), 3.34-3.91 (4H, m, 2 x $C\underline{H}_2O$), 3.63 (2H, t, *J* 6.6, $C\underline{H}_2$OH), 4.58 (1H, t, *J* 3.4, OC$\underline{H}$O); δ_C 19.69, 25.52, 25.68, 26.22, 29.18, 29.33, 30.81, 32.69, 62.35 ($\underline{C}H_2$OH), 62.90 (O$\underline{C}H_2CH_2$), 67.62 (O$\underline{C}H_2$), 98.86 (O$\underline{C}$HO); *m/z* 216 (M$^+$, 1%), 215 (9), 199 (2), 115 (6), 101 (19), 97 (23), 85 (100), and 55 (40). Found (M-1)$^+$, 215.1643 $C_{12}H_{23}O_3$ requires (M-1)$^+$, 215.1647.

7-Hydroxyheptanal (147)[107]

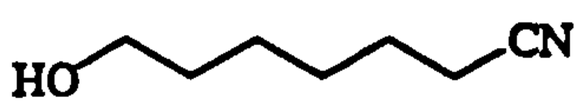


(147)

Aleuritic acid (157) (10 g, 32.8 mmol) was added with vigorous stirring to a solution of sodium hydroxide (1.33 g, 33.3 mmol) in water (40 cm^3). The resulting turbid solution

was treated with chloroform (40 cm^3), and then sodium periodate (8.43 g, 39.4 mmol) was added in portions at 35-40 °C over 10 minutes. The mixture was stirred at room temperature for a further 15 minutes, then filtered, and the filter cake was washed with chloroform (40 cm^3). The aqueous layer was extracted with chloroform (4 x 50 cm^3). The combined organic extracts were stirred for 1.25 h. with a mixture of saturated sodium hydrogen carbonate (90 cm^3) and sodium carbonate (60 cm^3). The organic layer was evaporated *in vacuo* to yield 7-hydroxyheptanal (147) (3.56 g, 83%) as a white crystalline solid. This was used without further purification. mp. 65-66 °C (lit.,[107] 65-67 °C); υ_{max} 3411, 1729 cm^{-1}; δ_H 1.33-1.91 (8H, m, 4 x $C\underline{H}_2$), 2.45 (2H, t, *J* 7.4, $C\underline{H}_2CHO$), 2.72 (1H, br s, O<u>H</u>), 3.60 (2H, t, *J* 7.2, $C\underline{H}_2OH$), 9.70 (1H, t, *J* 1.8, C<u>H</u>O).

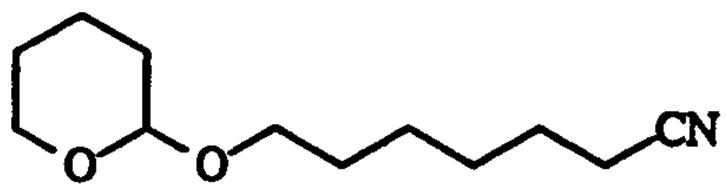
7-Hydroxyheptanitrile (159)



(159)

To a stirred solution of 6-bromo-1-hexanol (158) (2.03 g, 11.2 mmol) in distilled methanol (5 cm^3) was added sodium cyanide (0.67 g, 13.7 mmol) in distilled water (15 cm^3). The mixture was heated to reflux for six hours. After cooling this mixture in an ice-bath, the mixture was extracted with dichloromethane (5 x 25 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 50% ethyl acetate/petroleum ether 40-60 °C) yielded **7-hydroxyheptanitrile (159)** (0.98 g, 69%) as a colourless oil (R_f=0.3). υ_{max} 3402, 2245 cm^{-1}; δ_H 1.27-1.79 (8H, m, 4 x $C\underline{H}_2$), 1.83 (1H, br s, O<u>H</u>), 2.36 (2H, t, *J* 7.2, $C\underline{H}_2CN$), 3.64 (2H, t, *J* 6.4, $C\underline{H}_2OH$); δ_C 17.08 24.99, 25.30, 28.45, 32.32, 62.54 ($\underline{C}H_2OH$), 119.82 (<u>C</u>N); *m/z* 127 (M^+, 7%), 126 ($(M-1)^+$, 4), 110 ($(M-OH)^+$, 36), 83 (68), 82 (48), 69 (69), 68 (27), and 55 (100). Found MH^+, 128.1078 $C_7H_{14}NO$ requires MH^+, 128.1075.

7-(1'-Tetrahydropyran-2-yl)oxyheptanitrile (160)

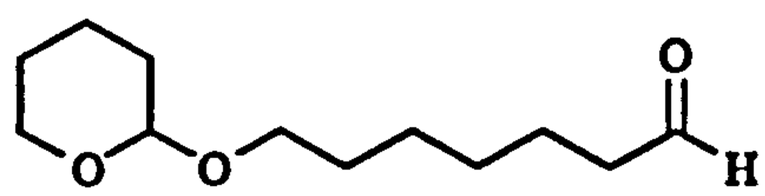


(160)

To 7-hydroxyheptanitrile (159) (0.66 g, 5.20 mmol) was added concentrated hydrochloric acid (0.03 cm^3), and the solution was heated to 50 °C. Then dihydropyran (0.73 g, 8.68 mmol) was added as a concentrated solution dropwise. The mixture was

stirred for 0.5 h., and then the temperature was increased to 80 °C, and was stirred overnight. The mixture was cooled in ice, and then extracted with benzene (2 x 30 cm^3). The combined organic extracts were washed with saturated sodium hydrogen carbonate (30 cm^3), and brine (30 cm^3). The organic extracts were dried over sodium sulphate, filtered, and concentrated *in vacuo*. Purification by Kugelrohr distillation (225 °C, 2 mmHg) yielded **7-(1'-tetrahydropyran-2-yl)oxyheptanitrile (160)** (0.98 g, 89%) as a colourless oil. υ_{max} 2244 cm^{-1}; δ_H 1.31-1.92 (14H, m, 7 x C$\underline{H}_2$), 2.36 (2H, t, *J* 6.8, C$\underline{H}_2$CN), 3.41-3.89 (4H, m, 2 x C$\underline{H}_2$O), 4.65 (1H, q, *J* 3.6, OC$\underline{H}$O); δ_C 17.11, 19.75, 23.41, 25.37, 25.52, 28.51, 29.46, 30.80, 62.52 (O$\underline{C}H_2CH_2$), 67.31 ($\underline{C}H_2O$), 99.00 (O$\underline{C}$HO), 119.83 ($\underline{C}$N); *m/z* 211 (M^+, 1%), 210 (M^+-1, 3), 138 (3), 110 (49), 85 (100), 83 (16), 69 (20), and 55 (20). Found MH^+, 212.1653 $C_{12}H_{22}O_3$ requires MH^+, 212.1650.

7-(1'-Tetrahydropyran-2-yl)oxyheptanal (154)[107,138,139]



(154)

(a) Via PDC oxidation of 7-[(tetrahydropyran-2-yl)oxy]heptan-1-ol (156)

To a stirred solution of pyridinium dichromate (0.19 g, 0.55 mmol) in freshly distilled dichloromethane (25 cm^3), with some dried, ground molecular sieves (3A), was added 7-[(tetrahydropyran-2-yl)oxy]heptan-1-ol (156) (0.06 g, 0.28 mmol), as a solution in freshly distilled dichloromethane (2 cm^3), under a nitrogen atmosphere. The resulting solution was left for 5 hours, then filtered through florisil and Celite. The filtrate was washed with HCl (2M, 25 cm^3), saturated sodium hydrogen carbonate (25 cm^3), and brine (25 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered, and concentrated *in vacuo* to yield 7-(1'-tetrahydropyran-2-yl)oxyheptanal (154) (0.05 g, 84%) as a pale yellow oil. This was used without further purification. υ_{max} 1725 cm^{-1}; δ_H 1.33-1.81 (14H, m, 7 x C$\underline{H}_2$), 2.40 (2H, t, *J* 7.4, C$\underline{H}_2$CHO), 3.31-3.88 (4H, m, 2 x C$\underline{H}_2$O), 4.54 (1H, t, *J* 3.5, OC$\underline{H}$O), 9.73 (1H, t, *J* 1.8, C$\underline{H}$O); δ_C 19.61, 21.89, 25.36, 25.87, 28.91, 29.42, 30.73, 43.68, 62.16 (O$\underline{C}H_2CH_2$), 67.29 ($\underline{C}H_2O$), 98.80 (O$\underline{C}$HO), 202.61 ($\underline{C}$HO); *m/z* 215 (MH^+, 8%), 213 (2), 199 (1), 185 (1), 157 (2), 101 (17), 85 (100), and 55 (19). Found MH^+, 215.1646 $C_{12}H_{23}O_3$ requires MH^+, 215.1647.

(b) Via Swern oxidation of 7-[(tetrahydropyran-2-yl)oxy]heptan-1-ol (156)[138]

To a stirred solution of oxalyl chloride (0.26 cm^3, 2.98 mmol) in dichloromethane (15 cm^3) at -78 °C under a dry nitrogen atmosphere was added a solution of dimethylsulphoxide (0.44 cm^3, 6.20 mmol) in dichloromethane (15 cm^3). After 5 minutes, 7-[(tetrahydropyran-2-yl)oxy]heptan-1-ol (156) (0.50 g, 2.31 mmol) in dichloromethane (5 cm^3) was added. The solution was allowed to warm to room temperature. Triethylamine (1 cm^3, 7.17 mmol) was added, and a white precipitate formed. The resultant mixture was stirred at room temperature for 2 hours, then was quenched with water (20 cm^3), and extracted with dichloromethane (4 x 40 cm^3). The organic extracts were washed with brine (75 cm^3), and were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield 7-(1'-tetrahydropyran-2-yl)oxyheptanal (154) (0.36 g, 73%) as a pale yellow oil. This was used without further purification. Spectral data as before.

(c) Via DIBAL reduction of 7-(1'-tetrahydropyran-2-yl)oxyheptanitrile (160)[107]

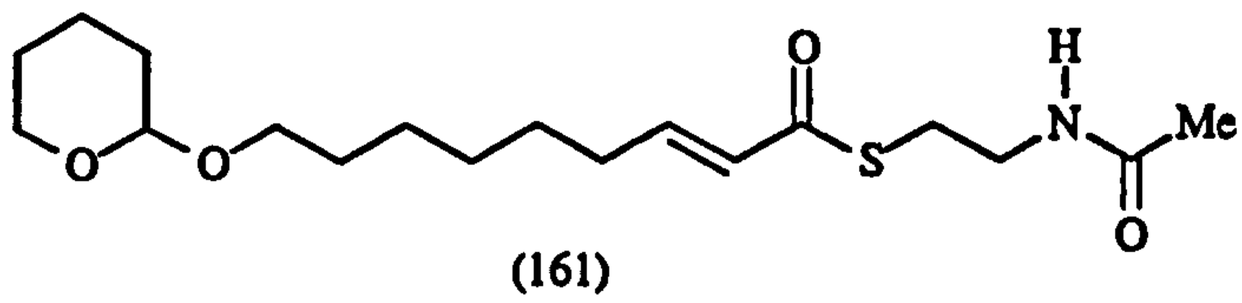
To a stirred solution of 7-(1'-tetrahydropyran-2-yl)oxyheptanitrile (160) (0.39 g, 1.9 mmol) in distilled ether (10 cm^3) under a nitrogen atmosphere at room temperature was added DIBAL-H (1M in toluene, 2.8 cm^3, 2.8 mmol). The mixture was stirred for 30 minutes. Ice-cold sulphuric acid (2M, 15 cm^3) was added, and the mixture was stirred for 45 minutes. It was then heated at 30 °C for 30 minutes. After this time, the solution was saturated with sodium chloride, and extracted with diethyl ether (3 x 20 cm^3). The combined organic extracts were washed with saturated sodium hydrogen carbonate (20 cm^3), brine (20 cm^3), and then dried over sodium sulphate. They were filtered and concentrated *in vacuo* to yield 7-(1'-tetrahydropyran-2-yl)oxyheptanal (154) (0.29 g, 73%) as a pale yellow oil. This was used without further purification. This was used without further purification. Spectral data as before.

(d) Via protection of 7-hydroxyheptanal (147)[107,139]

To 7-hydroxyheptanal (147) (2.36 g, 18.2 mmol) was added freshly distilled diethyl ether (35 cm^3). Dihydropyran (1.68 g, 20.0 mmol) in diethyl ether (5 cm^3) was added dropwise. pTSA (0.07 g, 0.37 mmol) was added, and the solution was stirred at room temperature for 24 hours. The mixture was then extracted with diethyl ether (2 x 50 cm^3). The combined organic extracts were washed with sodium hydroxide solution (2M, 40

cm^3), water (40 cm^3) and brine (40 cm^3). They were then dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield 7-(1'-tetrahydropyran-2-yl)oxyheptanal (154) (3.03 g, 78%) as a pale yellow oil. This was used without further purification. Spectral data as before.

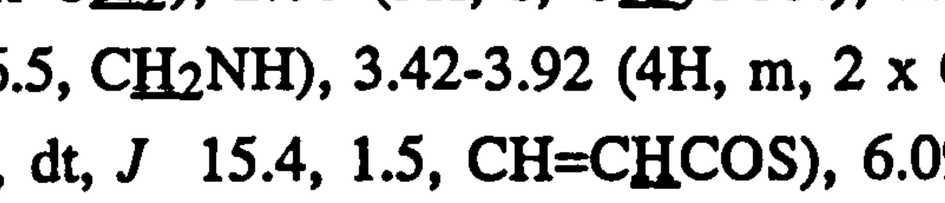
S-2-(Acetylamino)ethyl 9-(1'-tetrahydropyran-2-yl)oxynon-(2*E*)-enethioate (161) (N-acetylcysteamine thioester of 9-(1'-tetrahydropyran-2-yl)oxynon-(2*E*)-enoic acid)



(161)

To 7-(1'-tetrahydropyran-2-yl)oxyheptanal (154) (0.07 g, 0.33 mmol) in chloroform (20 cm^3) was added the N-acetylcysteamine thioester (151) of triphenylphosphorane acetate (0.13 g, 0.31 mmol), and the reaction mixture was heated to reflux, under a nitrogen atmosphere, for 4 days. The solvent was removed *in vacuo* to yield **S-2-(acetylamino)ethyl 9-(1'-tetrahydropyran-2-yl)oxynon-(2*E*)-enethioate (161)** (0.10 g, 90%) as a yellow oil (R_f=0.35). υ_{max} 3283, 1717, 1656, 1551 cm^{-1}; δ_H 1.34-1.71 (16H, m, 8 x $C\underline{H}_2$), 2.00 (3H, s, $C\underline{H}_3CON$), 2.66 (2H, dt, *J* 8.5, 6.5, $C\underline{H}_2S$), 3.39 (2H, q, *J* 6.5, $C\underline{H}_2NH$), 3.42-3.92 (4H, m, 2 x $C\underline{H}_2O$), 4.57 (1H, dd, *J* 3.5, OC<u>H</u>O), 5.80 (1H, dt, *J* 15.4, 1.5, CH=C<u>H</u>COS), 6.09 (1H, br s, N<u>H</u>), 6.95 (1H, m, C<u>H</u>=CHCOS); δ_C 19.66, 24.55 ($\underline{C}H_2NH$), 25.44, 25.98, 26.43, 27.92, 28.90 ($\underline{C}H_2S$), 29.57, 30.74, 32.04, 42.61 ($\underline{C}H_2CON$), 62.33 ($O\underline{C}H_2CH_2$), 67.51 ($\underline{C}H_2O$), 98.84 (O<u>C</u>HO), 128.40 (CH=<u>C</u>HCOS), 131.64 (<u>C</u>H=CHCOS), 170 .16 (<u>C</u>ON), 191.42 (<u>C</u>OS). *m/z* (CI) 274 ($(M\text{-}THP)^+$, 39%), 237 (85), 155 (24), 120 (70), 118 (100), and 86 (60).

Ethyl 9-(1'-tetrahydropyran-2-yl)oxynon-(2*E*)-enoate (163)

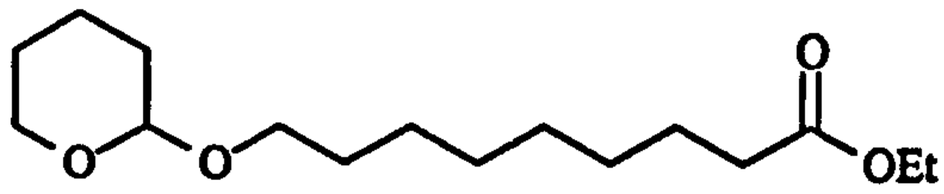


(163)

Sodium hydride (60% dispersion oil, 0.06 g, 1.50 mmol) was stirred in THF (15 cm^3) under a nitrogen atmosphere. Ethyl phosphonoacetate (162) (0.27 g, 1.21 mmol) in distilled THF (3 cm^3) was added dropwise at 0 °C, and stirred for 0.5 h. Then 7-(1'-

tetrahydropyran-2-yl)oxyheptanal (154) (0.23 g, 1.07 mmol) in THF (3 cm^3) was added dropwise at 0 °C. The resulting solution was stirred overnight at room temperature. The reaction mixture was then quenched with distilled water (100 cm^3), and was extracted with ethyl acetate (3 x 75 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 10% ethyl acetate/ petroleum ether 40-60 °C) yielded **ethyl 9-(1'-tetrahydropyran-2-yl)oxynon-(2*E*)-enoate (163)** (0.19 g, 62%) as a pale yellow oil (R_f=0.3). υ_{max} 1724, 1650 cm^{-1}; δ_H 1.29 (3H, t, *J* 7.0, $C\underline{H}_3CH_2O$), 1.36-1.95 (14H, m, 7 x $C\underline{H}_2$), 2.20 (2H, q, *J* 7.4, $C\underline{H}_2CH{=}CHCO_2Et$), 3.33-3.91 (4H, m, 2 x $C\underline{H}_2O$), 4.18 (2H, q, *J* 7.0, $CH_3C\underline{H}_2O$), 4.58 (1H, dd, *J* 3.5, $OC\underline{H}O$), 5.80 (1H, dt, *J* 15.7, 1.5, $CH{=}C\underline{H}CO_2Et$), 6.96 (1H, m, $C\underline{H}{=}CHCO_2Et$); δ_C 14.25 ($\underline{C}H_3CH_2O$), 19.71, 26.17, 27.92, 28.94, 29.30, 29.67, 30.75, 32.08, 60.11 ($CH_3\underline{C}H_2O$), 62.36 ($\underline{C}H_2O$), 67.62 ($O\underline{C}H_2$), 98.83 ($O\underline{C}HO$), 121.24 ($CH{=}\underline{C}HCO_2Et$), 149.31 ($\underline{C}H{=}CHCO_2Et$), 166.91 ($\underline{C}O_2Et$); *m/z* (CI) 283 ($(M-1)^+$, 1%), 279 (5), 155 (21), 109 (17), 97 (17), 85 (100), 67 (10), and 57 (24). Found $(M-1)^+$, 283.1915 $C_{16}H_{27}O_4$ requires $(M-1)^+$, 283.1909.

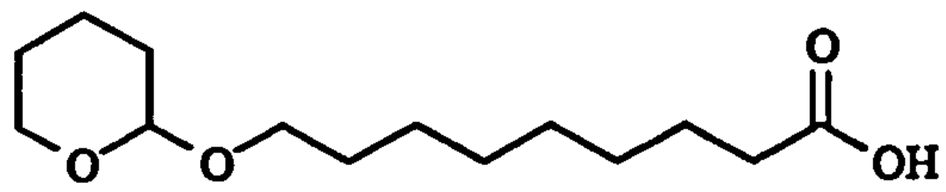
Ethyl 9-(1'-tetrahydropyran-2-yl)oxynonanoate (164)



(164)

To a solution of ethyl 9-(1'-tetrahydropyran-2-yl)oxynon-(2E)-enoate (163) (0.39 g, 1.37 mmol) in methanol (5 cm^3) was added palladium on activated charcoal (37mg), and the reaction mixture was stirred at room temperature under a hydrogen atmosphere for 4 hours. The solution was then filtered, in order to remove the catalyst, and the solvent was removed *in vacuo* to yield **ethyl 9-(1'-tetrahydropyran-2-yl)oxynonanoate (164)** (0.28 g, 71%) as a pale yellow oil. δ_H 1.28 (3H, t, *J* 7.0, $C\underline{H}_3CH_2O$), 1.36-1.95 (18H, m, 7 x $C\underline{H}_2$), 2.26 (2H, t, *J* 6.4, $C\underline{H}_2CO_2Et$), 3.33-3.91 (4H, m, 2 x $C\underline{H}_2O$), 4.17 (2H, q, *J* 7.0, $CH_3C\underline{H}_2O$), 4.58 (1H, t, *J* 3.5, $OC\underline{H}O$); *m/z* (CI) 287 (MH^+, 2%), 242 (32), 240 (26), 200 (14), and 85 (100).

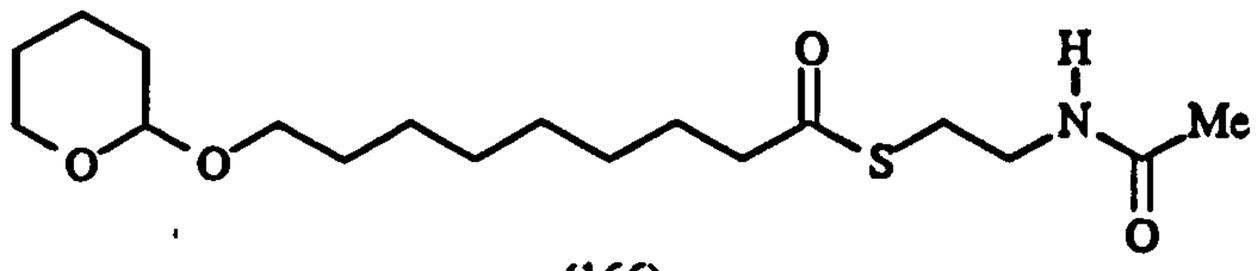
9-(1'-Tetrahydropyran-2-yl)oxynonanoic acid (165)



(165)

To a stirred solution of ethyl 9-(1'-tetrahydropyran-2-yl)oxynonanoate (164) (0.5 g, 1.75 mmol) was added sodium hydroxide solution (2M, 1.0 cm^3, 2.00 mmol), together with the phase transfer catalyst, tetrabutyl ammonium bromide (10mg). The mixture was stirred at room temperature overnight. Hydrochloric acid (2M, 30 cm^3) was added, the solution was stirred at room temperature for 3 hours, and was then extracted with diethyl ether (5 x 50 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield **9-(1'-tetrahydropyran-2-yl)oxynonanoic acid (165)** (0.35 g, 78%) as a colourless oil. This was used without further purification. υ_{max} 3400, 1708 cm^{-1}; δ_H 1.32-1.86 (18H, m, 9 x $C\underline{H}_2$), 2.35 (2H, t, *J* 7.4, $C\underline{H}_2CO_2H$), 3.34-3.92 (4H, m, 2 x $C\underline{H}_2O$), 4.58 (1H, t, *J* 3.5, $OC\underline{H}O$), 10.42 (1H, br s, $CO_2\underline{H}$); δ_C 19.62, 24.62, 25.45, 26.12, 28.96, 29.12, 29.21, 29.69, 30.73, 33.93 ($\underline{C}H_2CO_2H$), 62.91 ($\underline{C}H_2O$), 67.62 ($O\underline{C}H_2$), 98.80 ($O\underline{C}HO$), 179.26 ($\underline{C}O_2H$) ; *m/z* (CI) 258 (M^+, 4%), 175 (11), 174 (6), 157 (8), 139 (16), 121 (3), 85 (100), and 57 (6). Found MH^+, 259.1918 $C_{14}H_{27}O_4$ requires MH^+, 259.1909.

S-2-(Acetylamino)ethyl 9-(1'-tetrahydropyran-2-yl)oxynonanethioate (166) (N-acetylcysteamine thioester of 9-(1'-tetrahydropyran-2-yl)oxynonanoic acid)

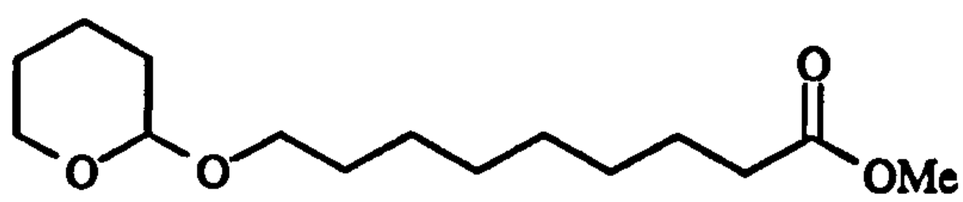


(166)

Freshly prepared N-acetylcysteamine (73) (0.47 g, 3.95 mmol) in dichloromethane (4 cm^3) was stirred at 0 °C, under a nitrogen atmosphere. Dicyclohexylcarbodiimide (83) (0.80 g, 3.88 mmol) in dichloromethane (4 cm^3) was added, followed by 4-dimethylaminopyridine (84) (0.024 g, 0.20 mmol), in dichloromethane (2 cm^3). 9-(1'-tetrahydropyran-2-yl)oxynonanoic acid (165) (0.90 g, 3.49 mmol) in dichloromethane (4 cm^3) was then added dropwise. An immediate white precipitate of dicyclohexylurea formed. The solution was left at 0 °C for 2 h., and was then allowed to warm to room temperature overnight. The resulting solution was filtered through Celite to remove the precipitate. To the filtrate was added saturated ammonium chloride (50 cm^3), and was extracted with dichloromethane (2 x 20 cm^3). The combined organic extracts were dried

over magnesium sulphate, filtered and concentrated *in vacuo* to yield the **S-2-(acetylamino)ethyl 9-(1'-tetrahydropyran-2-yl)oxynonanethioate (166)** (1.15 g, 93%) as a colourless oil. This was used without further purification. υ_{max} 3356, 1693, 1665 cm^{-1}; δ_H 1.31-1.82 (18H, m, 9 x C$\underline{H}_2$), 2.62 (2H, t, *J* 7.6, C$\underline{H}_2$COS), 3.06 (2H, t, *J* 7.4, C$\underline{H}_2$S), 3.30-3.91 (4H, m, 2 x C$\underline{H}_2$O), 3.41 (2H, t, *J* 7.4, C$\underline{H}_2$NH), 4.58 (1H, t, *J* 3.5, OC$\underline{H}$O), 5.93 (1H, br s, N$\underline{H}$); δ_C 19.75, 23.24 (C$\underline{H}_2$NH), 24.71, 25.50, 26.16, 28.44 (C$\underline{H}_2$S), 28.85, 29.22, 29.71, 30.79, 33.97, 34.92, 39.78 (C$\underline{H}_3$CON), 62.42 (C$\underline{H}_2$O), 67.64 (OC$\underline{H}_2$), 98.92 (OC$\underline{H}$O), 170.28 (C$\underline{O}$N), 200.27 (C$\underline{O}$S); *m/z* (CI) 360 (MH^+, 4%), 276 (90), 139 (6), 120 (100), 85 (31), and 57 (10). Found MH^+, 360.2214 $C_{18}H_{34}NO_4S$ requires MH^+, 360.2209.

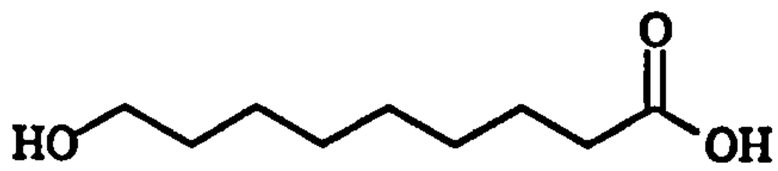
Treatment of the N-acetylcysteamine thioester (166) of 9-(1'-tetrahydropyran-2-yl)oxynonanoic acid, in methanol, with amberlyst-15



(167)

To a solution of the N-acetylcysteamine thioester (166) of 9-(1'-tetrahydropyran-2-yl)oxynonanoic acid (1.05 g, 2.92 mmol) in freshly distilled methanol (20 cm^3) was added a catalytic amount of amberlyst-15. The resulting solution was then stirred at room temperature for 18 h. The mixture was then filtered, and the solvent was concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 25% ethyl acetate, petroleum ether 40-60 °C) yielded **methyl 9-(1'-tetrahydropyran-2-yl)oxynonanoate (167)** (0.61 g, 77%) as a colourless oil (R_f=0.7). δ_H 1.31-1.88 (18H, m, 9 x C$\underline{H}_2$), 2.31 (2H, t, *J* 7.5, C$\underline{H}_2$$CO_2$Me), 3.33-3.91 (4H, m, 2 x C$\underline{H}_2$O), 3.67 (3H, s, OC$\underline{H}_3$), 4.58 (1H, t, *J* 3.5, OC$\underline{H}$O); δ_C 19.72, 24.94, 25.51, 26.18, 29.07, 29.23, 29.44, 29.72, 30.79, 34.10, 51.46 (OC$\underline{H}_3$), 62.36 (C$\underline{H}_2$O), 67.65 OC$\underline{H}_2$), 98.86 (OC$\underline{H}$O), 174.33 (C$\underline{O}_2$Me). *m/z* (CI) 256 ($(M-Me)^+$, 16%), 237 (100), 225 (42), 195 (32), 125 (12), 120 (40), 118 (82), and 86 (61).

9-Hydroxynonanoic acid (69)

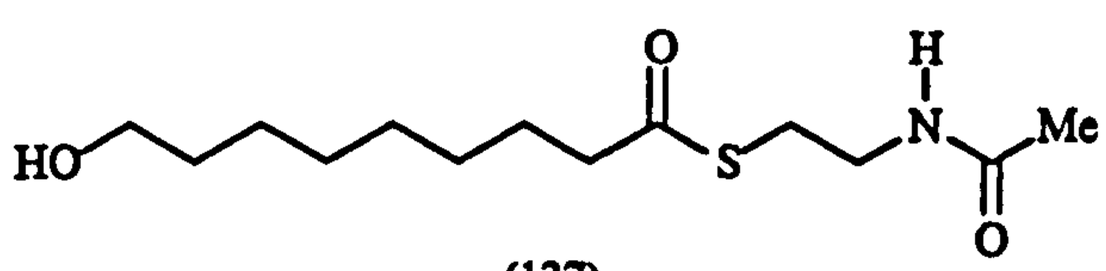


(69)

To a stirred solution of 9-(1'-tetrahydropyran-2-yl)oxynonanoic acid (165) (0.15 g, 0.58

mmol) was added freshly distilled methanol (15 cm^3), and a catalytic amount of amberlyst-15. The resulting solution was stirred overnight at room temperature. This was then filtered, and the solvent removed *in vacuo* to yield **9-hydroxynonanoic acid (69)** (0.05 g, 49%) as a white crystalline solid. Spectral data as before.

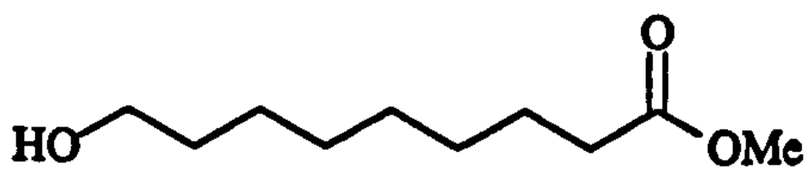
S-2-(Acetylamino)ethyl 9-hydroxynonanethioate (137) (N-acetylcysteamine thioester of 9-hydroxynonanoic acid)



(137)

Freshly prepared N-acetylcysteamine (73) (0.04 g, 0.34 mmol) in dichloromethane (5 cm^3) was stirred at 0 °C, under a nitrogen atmosphere. Dicyclohexylcarbodiimide (83) (0.05 g, 0.26 mmol) in dichloromethane (1 cm^3) was added, followed by 4-dimethylaminopyridine (84) (0.003 g, 0.02 mmol), in dichloromethane (0.2 cm^3). This was stirred for 10 minutes, and then followed by addition of 9-hydroxynonanoic acid (69) (0.04 g, 0.23 mmol) in dichloromethane (1 cm^3). The solution was left at 0 °C for 2 h., and was then allowed to warm to room temperature overnight. The solution was quenched with saturated ammonium chloride (50 cm^3), and was extracted with dichloromethane (2 x 20 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield the **S-2-(acetylamino)ethyl 9-hydroxynonanethioate (137)** (0.05 g, 79%) as a colourless oil (R_f=0.4). δ_H 1.31-1.66 (12H, m, 6 x $C\underline{H}_2$), 1.97 (3H, s, $C\underline{H}_3CO$), 2.57 (2H, t, *J* 7.4, $C\underline{H}_2COS$), 2.80 (1H, br s, O<u>H</u>), 3.02 (2H, t, *J* 6.4, $C\underline{H}_2S$), 3.39 (2H, t, *J* 6.4, $C\underline{H}_2NH$) , 3.63 (2H, t, *J* 6.6, $C\underline{H}_2OH$), 6.32 (1H, br s, N<u>H</u>); δ_C 23.12 ($\underline{C}H_2S$), 25.58, 25.63, 28.35 ($\underline{C}H_2NH$), 28.73, 29.10, 32.65, 39.53 ($\underline{C}H_2COS$), 39.70 ($\underline{C}H_3CON$), 44.06 ($\underline{C}H_2CH_2OH$), 62.76 ($\underline{C}H_2OH$), 170.67 ($\underline{C}ON$), 200.35 ($\underline{C}OS$); *m/z* (CI) 276 (MH^+, 59%), 237 (42), 195 (10), 157 (11), 139 (28), 120 (100), and 86 (28). Found MH^+, 276.1641 $C_{13}H_{26}NO_3S$ requires MH^+, 276.1633.

Methyl-9-hydroxynonanoate (168)[48]

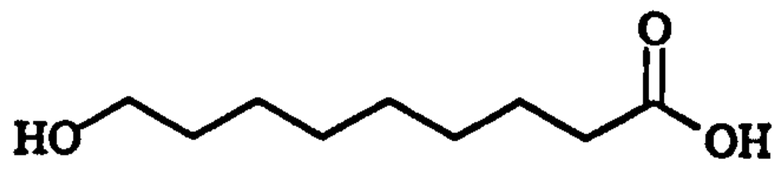


(168)

To a stirred solution of methyl-9-(1'-tetrahydropyran-2-yl)oxynonanoate (167) (0.20 g,

0.74 mmol) was added freshly distilled methanol (10 cm^3), and a catalytic amount of amberlyst-15. The resulting solution was stirred at room temperature overnight. This was then filtered, and the solvent removed *in vacuo*. Purification by flash column chromatography (SiO_2, 50% ethyl acetate/ petroleum ether 40-60 °C) yields methyl-9-hydroxynonanoate (168) (0.09 g, 65%) as a colourless oil (R_f=0.5). δ_H 1.21-1.89 (12H, m, 6 x $C\underline{H}_2$), 2.31 (2H, t, *J* 7.4, $C\underline{H}_2CO_2Me$), 2.63 (1H, br s, O<u>H</u>), 3.61 (2H, t, *J* 6.5, $C\underline{H}_2OH$), 3.67 (3H, s, $OC\underline{H}_3$); δ_C 24.91, 25.66, 29.09, 29.19, 29.71, 32.73 ($\underline{C}H_2CH_2OH$), 34.08 ($\underline{C}H_2CO_2Me$), 51.49 ($O\underline{C}H_3$), 62.36 ($\underline{C}H_2OH$), 174.38 ($\underline{C}O_2Me$); *m/z* (CI) 189 (MH+, 30%), 171 (40), 157 (58), 139 (83), 129 (10), 111 (37), 85 (100), and 69 (21).

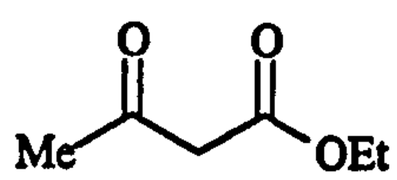
9-Hydroxynonanoic acid (69)



(69)

Methyl-9-hydroxynonanoate (168) (0.20 g, 0.84 mmol) in 1M sodium hydroxide solution (1.0 cm^3, 1.00 mmol) was stirred at room temperature overnight. Hydrochloric acid (1M, 2 cm^3) was then added, the solution was stirred at room temperature for 0.5 h., and then extracted with diethyl ether (4 x 10 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield **9-hydroxynonanoic acid (69)** (0.09 g, 62%) as a white crystalline solid. Spectral data as before.

Ethyl 3-oxobutanoate (172) (Ethyl acetoacetate)[71]

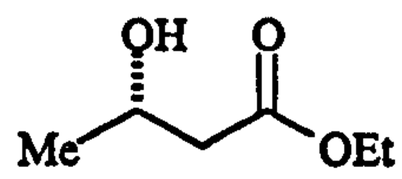


(172)

To THF (50 cm^3) was added hexamethyldisilazane (6.62 g, 41.0 mmol) at -78 °C under a nitrogen atmosphere, followed by n-butyl lithium (2.5M, 14.8 cm^3, 37.0 mmol). The solution was warmed to 0 °C over 15 minutes, and then cooled again to -78 °C over a further 15 minutes. Ethyl acetate (101) (2.00 g, 22.7 mmol) in THF (3 cm^3) was added dropwise. This solution was left stirring for 0.5 h. at -78 °C. Acetyl chloride (1.97 g, 25.1 mmol) in freshly distilled THF (3 cm^3) was added dropwise at -78 °C, the mixture was stirred for 45 minutes, and then warmed to room temperature. The reaction mixture

was quenched with hydrochloric acid solution (5%), and stirred for 5 minutes. The THF was removed *in vacuo*, and the residue was extracted with ethyl acetate (3 x 75 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered, and concentrated *in vacuo*. Purification by Kugelrohr distillation (58 °C, 5 mmHg) yielded ethyl acetoacetate (172) (1.84 g, 62%) as a colourless oil. lit.,[136] bp. 54 °C at 5 mmHg; υ_{max} 1732, 1649 cm^{-1}; δ_H 1.29 (3H, t, *J* 7.2, $C\underline{H}_3CH_2$), 2.28 (3H, s, $C\underline{H}_3CO$), 3.46 (2H, s, $COC\underline{H}_2CO$), 4.21 (2H, q, *J* 7.2, $CH_3C\underline{H}_2$); δ_C 14.2 ($\underline{C}H_3CH_2O$), 30.1 ($\underline{C}H_3CO$), 50.1 ($OC\underline{C}H_2CO$), 61.3 ($CH_3\underline{C}H_2O$), 167.1 ($\underline{C}O_2Et$), 200.7 ($CH_3\underline{C}O$); *m/z* (CI) 131 (MH^+, 35%), 130 (18), 85 (74), 84 (11), and 65 (100).

Ethyl (3*S*)-3-hydroxybutanoate (173)[112]

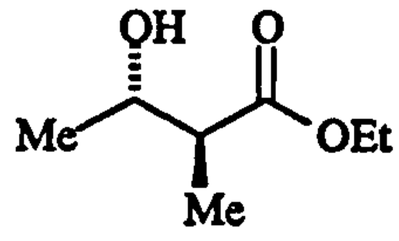


(173)

To each of ten conical flasks (250 cm^3) were added glucose (20 g), distilled water (100 cm^3), and dried Baker's yeast (*Sacchoromyces cerevisiae*) (10 g). These flasks were incubated for 1 hour at 35 °C at 150rpm. To each flask was added ethyl acetoacetate (172) (1 g), and the flasks were shaken for 48 hours at 30 °C at 250rpm. After this time, Celite (10 g) was added to each flask, and the mixture was filtered under reduced pressure. The filtrate was then extracted with diethyl ether (5 x 250 cm^3). The combined organic extracts were washed with brine (3 x 250 cm^3), and then dried over magnesium sulphate, filtered, and concentrated *in vacuo*. Purification by Kugelrohr distillation (50 °C, 2 mmHg) yielded ethyl (3*S*)-3-hydroxybutanoate (173) (5.45 g, 53%) as a colourless oil. $[\alpha]_D$ +32.0 (*c* 2.19 $CHCl_3$), [lit.,[112] +41.5 (*c* 1.0, $CHCl_3$)]; υ_{max} 3450, 1728 cm^{-1}; δ_H 1.23 (3H, d, *J* 6.4, $C\underline{H}_3CH(OH)$), 1.28 (3H, d, *J* 7.2, $C\underline{H}_3CH_2O$), 2.46 (2H, m, $C\underline{H}_2CO_2$), 3.46 (1H, br s, O<u>H</u>), 4.17 (2H, q, *J* 7.2, $CH_3C\underline{H}_2O$), 4.22 (1H, m, $CH_3C\underline{H}(OH)$); δ_C 14.21 ($\underline{C}H_3CH_2O$), 22.43 ($\underline{C}H_3CH(OH)$), 42.74 ($\underline{C}H_2CO_2$), 60.71 ($CH_3\underline{C}H(OH)$), 64.29 ($CH_3\underline{C}H_2O$), 173.00 ($CH_2\underline{C}O_2$); *m/z* (CI) 133 (MH^+, 8%), 131 (3), 115 (31), 103 (6), 87 (51), 85 (75), 83 (100), and 73 (36).

N.B. This Baker's yeast reduction gave ethyl (3*S*)-3-hydroxybutanoate in 70% e.e., based on the values of optical purity obtained from the $[\alpha]_D$. Therefore, all subsequent steps, using this compound as a starting material can only be carried out, at best, in 70% e.e.

Ethyl (2*S*,3*S*)-3-hydroxy-2-methylbutanoate (179)[140]

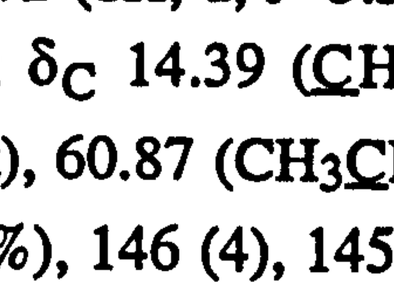


(179)

To a stirred solution of freshly distilled THF (20 cm^3), under a nitrogen atmosphere at -78 °C was added LDA (Aldrich Chemicals, 1.5M, 15.1 cm^3, 22.7 mmol). Ethyl (3*S*)-3-hydroxybutanoate (173) (1 g, 7.6 mmol) was added dropwise, as a solution in THF (5 cm^3). The mixture was stirred at -78 °C for 0.5 h., warmed to 0 °C, and then recooled to -78 °C. Methyl iodide (4.76 cm^3, 76.0 mmol) was then added dropwise, and the resulting solution was warmed to room temperature overnight. The reaction was quenched with saturated ammonium chloride solution (50 cm^3), and then extracted with diethyl ether (4 x 25 cm^3). The combined organic extracts were then dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 20% ethyl acetate/ petroleum ether 40-60 °C) yielded ethyl (2*S*,3*S*)-3-hydroxy-2-methylbutanoate (179) (0.60 g. 54%) as a colourless oil. $[\alpha]_D^{20}$ +20.4 (*c* 1.6, $CHCl_3$), [lit.,[140] +19.1 (*c* 1.3 $CHCl_3$)]; υ_{max} 3450, 1730 cm^{-1}; δ_H 1.19 (3H, d, *J* 7.2, $C\underline{H}_3CH$), 1.22 (3H, d, *J* 7.2, $C\underline{H}_3CH$),1.28 (3H, t, *J* 7.2, $C\underline{H}_3CH_2O$), 2.44 (1H, m, $C\underline{H}(CH_3)CO_2Et$), 2.72 (1H, d, *J* 5.5, O<u>H</u>), 3.88 (1H, m, $CH_3C\underline{H}(OH)$), 4.18 (2H, q, *J* 7.2, $CH_3C\underline{H}_2O$); δ_C 14.39 ($\underline{C}H_3CH$), 14.45 ($\underline{C}H_3CH_2O$), 21.03 ($\underline{C}H_3CH$), 47.19 ($\underline{C}H(CH_3)CO_2Et$), 60.87 ($CH_3\underline{C}H_2O$), 69.69 ($CH_3\underline{C}H(OH)$), 176.21 ($\underline{C}O_2Et$); *m/z* (CI) 147 ($MH^+$, 11%), 146 (4), 145 (7), 129 (10), 101 (11), 85 (22), 57 (100), and 55 (63).

N.B. The Baker's yeast reduction gave *ethyl (3S)*-3-hydroxybutanoate in 70% e.e. Therefore, this compound is only, at best, synthesised *in 70%* e.e.

(2*S*,3*S*)-3-Hydroxy-2-methylbutanoic acid (180)



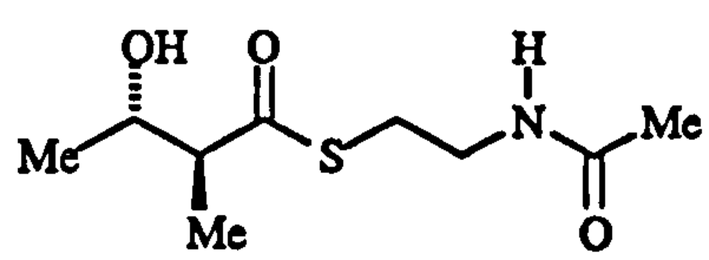
(180)

Ethyl (2*S*, 3*S*)-3-hydroxy-2-methylbutanoate (179) (0.50 g, 3.4 mmol) in sodium hydroxide solution (2M, 1.7 cm^3, 3.4 mmol) was stirred at room temperature for 18 hours. After this time, the solution was adjusted to pH 1.0 using hydrochloric acid

solution (2M). This was then extracted using ice-cold ethyl acetate (5 x 20 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield **(2*S*,3*S*)-3-hydroxy-2-methylbutanoic acid (180)** (0.37 g. 92%) as a pale yellow oil. This was used without further purification. $[\alpha]_D^{20}$ +17.4 (*c* 3.0, $CHCl_3$); υ_{max} 3393, 17.12 cm^{-1}; δ_H 1.21 (3H, d, *J* 7.2, $C\underline{H}_3CH$), 1.26 (3H, d, *J* 8.0, $C\underline{H}_3CH(OH)$), 2.49 (1H, m, $CH_3C\underline{H}$), 3.93 (1H, m, $CH_3C\underline{H}(OH)$), 6.37 (2H, br s, 2 x $O\underline{H}$); δ_C 13.70 ($\underline{C}H_3CH$), 19.99 ($\underline{C}H_3CH$), 46.69 ($\underline{C}H(CH_3)CO_2H$), 69.26 ($CH_3\underline{C}H(OH)$), 180.31 ($\underline{C}O_2H$); *m/z* (CI) 119 ($MH^+$, 31%), 117 (2), 101 (100), 83 (29), 73 (21), and 57 (24).

N.B. The Baker's yeast reduction gave ethyl (3*S*)-3-hydroxybutanoate in 70% e.e. Therefore, this compound is only, at best, synthesised in 70% e.e.

S-2-(Acetylamino)ethyl (2*S*,3*S*)-3-hydroxy-2-methylbutanethioate (169)
(N-acetylcysteamine thioester of (2*S*,3*S*)-3-hydroxy-2-methylbutanoic acid)



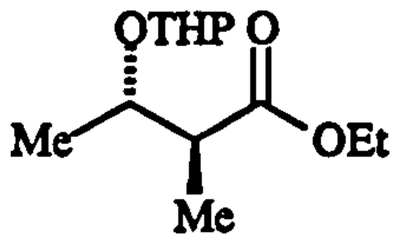
(169)

Freshly prepared N-acetylcysteamine (73) (0.49 g, 4.12 mmol) in dichloromethane (2 cm^3) was stirred at 0 °C, under a nitrogen atmosphere. Dicyclohexylcarbodiimide (83) (0.72 g, 3.49 mmol) in dichloromethane (2 cm^3) was added, followed by 4-dimethylaminopyridine (84) (0.02 g, 0.16 mmol), in dichloromethane (1 cm^3). (2*S*,3*S*)-3-hydroxy-2-methylbutanoic acid (180) (0.37 g, 3.14 mmol) in dichloromethane (2 cm^3) was then added dropwise. An immediate white precipitate of dicyclohexylurea formed. The solution was left at 0 °C for 3 hours, and was then allowed to warm to room temperature overnight. The resulting solution was filtered through Celite to remove the precipitate. The filtrate was washed with saturated ammonium chloride (10 cm^3), and was extracted with dichloromethane (2 x 10 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* . Purification by flash column chromatography (Al_2O_3, 99% ethyl acetate/1% methanol) yielded the **S-2-(acetylamino)ethyl (2*S*,3*S*)-3-hydroxy-2-methylbutanethioate (169)** (0.17 g, 25%) as a colourless oil. $[\alpha]_D^{20}$ +21.4 (*c* 2.9, $CHCl_3$); υ_{max} 3424, 3289, 1732, 1658 cm^{-1}; δ_H 1.20 (3H, d, *J* 7.0, $C\underline{H}_3CH$), 1.24 (3H, d, *J* 6.4, $C\underline{H}_3CH(OH)$), 1.73 (1H, br s, $O\underline{H}$), 1.97 (3H, s, $C\underline{H}_3CO$), 2.68 (1H, m, $CH_3C\underline{H}$), 3.05 (2H, m, $C\underline{H}_2S$), 3.45 (2H, m, $C\underline{H}_2N$), 3.95 (1H, m, $CH_3C\underline{H}(OH)$, 5.91 (1H, br s, $N\underline{H}$); δ_C 14.93 ($\underline{C}H_3CH$), 21.10 ($\underline{C}H_3CH(OH)$), 23.22 ($\underline{C}H_3CON$), 28.64 ($CH_3\underline{C}H$), 39.36 ($\underline{C}H_2S$), 55.91 ($\underline{C}H_2NH$), 69.99 ($CH_3\underline{C}H(OH)$), 170.53 ($\underline{C}ON$), 203.99 ($\underline{C}OS$); *m/z* (CI) 220

(MH$^+$, 100%), 219 (M$^+$, 1), 202 (21), 183 (20), 120 (93), 119 (26), 118 (52), and 101 (35). Found MH$^+$, 220.1010 $C_9H_{18}NO_3S$ requires MH$^+$, 220.1007.

N.B. The Baker's yeast reduction gave ethyl (3*S*)-3-hydroxybutanoate in 70% e.e. Therefore, this compound is only, at best, synthesised in 70% e.e.

Ethyl (2*S*,3*S*)-3-(1'-tetrahydropyran-2-yl)oxy-2-methylbutanoate (187)

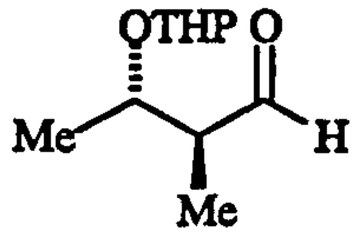


(187)

Ethyl (2*S*, 3*S*)-3-hydroxy-2-methylbutanoate (179) (0.80 g, 5.48 mmol) in dichloromethane (25 cm^3) was stirred with dihydropyran (0.55 cm^3, 6.03 mmol), and a catalytic amount of pTSA, was stirred overnight at room temperature, under a nitrogen atmosphere. The mixture was concentrated *in vacuo*, and dissolved in diethyl ether (50 cm^3). The organic layer was washed with water (30 cm^3), sodium hydrogen carbonate (30 cm^3), and brine (30 cm^3). The remaining aqueous layers were re-extracted with diethyl ether (2 x 50 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered, and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 10% ethyl acetate/petroleum ether 40-60 °C) yielded **ethyl (2*S*,3*S*)-3-(1'-tetrahydropyran-2-yl)oxy-2-methylbutanoate (187)** (1.20 g, 95%) as a colourless oil. υ_{max} 1737 cm^{-1}; δ_H 1.10 (1.5H, d, *J* 7.0, C$\underline{H}_3$CHCO), 1.15 (1.5H, d, *J* 7.2, C$\underline{H}_3$CH(OTHP)), 1.23 (1.5H, d, *J* 7.0, C$\underline{H}_3$CHCO), 1.25 (3H. t, *J* 7.0, C$\underline{H}_3$CH$_2$O), 1.29 (1.5H, d, *J* 7.2, C$\underline{H}_3$CH(OTHP)), 1.41-1.86 (6H, m, 3 x C$\underline{H}_2$), 2.58 (0.5H, dq, *J* 6.2, 6.2, CH$_3$C$\underline{H}$CO), 2.65 (0.5H, dq, *J* 6.2, 6.2, CH$_3$C$\underline{H}$CO), 3.55 (1H, m, CH$_3$C$\underline{H}$(OTHP)), 3.78-4.00 (2H, m, C$\underline{H}_2$O), 4.18 (2H, q, *J* 7.0, CH$_3$C$\underline{H}_2$O), 4.63 (O.5H, dd, *J* 3.5, OC$\underline{H}$O), 4.79 (O.5H, dd, *J* 3.5, OC$\underline{H}$O). *m/z* (CI) 185 ((M-OEt)$^+$, 62%), 183 (75), 129 (11), 101 (21), 85 (100), 71 (15), and 57 (15). Found MH$^+$, 185.1181 $C_{10}H_{17}O_3$ requires MH$^+$, 185.1178.

N.B. The Baker's yeast reduction gave ethyl (3*S*)-3-hydroxybutanoate in 70% e.e. Therefore, this compound is only, at best, synthesised in 70% e.e.

(2*S*,3*S*)-3-(1'-Tetrahydropyran-2-yl)oxy-2-methylbutanal (188)

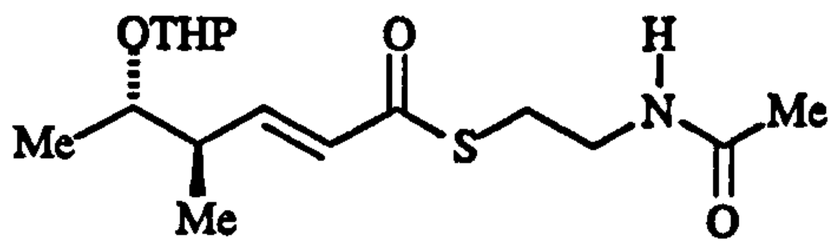


(188)

DIBAL-H solution in toluene (1M, 1 cm^3, 1.0 mmol) was added dropwise to a solution of ethyl (2*S*,3*S*)-3-(1'-tetrahydropyran-2-yl)oxy-2-methylbutanoate (187) (0.23 g, 1.0 mmol) in dry toluene (10 cm^3) at -78 °C under a nitrogen atmosphere. The reaction was monitored by TLC, and was found to be complete after 1 hour. The reaction mixture was quenched with methanol (2 cm^3) at -78 °C, and then allowed to warm to room temperature. Saturated ammonium chloride solution (1 cm^3) was added and stirred vigorously for 10 minutes. The precipitated alumina was then removed by filtering through Celite, which was then washed with toluene. The filtrate was washed with water (20 cm^3) and brine (20 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered, and concentrated *in vacuo* to yield **(2*S*,3*S*)-3-(1'-tetrahydropyran-2-yl)oxy-2-methylbutanal (188)** (0.16 g, 86%) as a pale yellow oil. This was used without further purification. υ_{max} 1727 cm^{-1}; δ_H 1.07 (1.5H, d, *J* 6.4, $C\underline{H}_3CHCO$), 1.11 (1.5H, d, *J* 7.2, $C\underline{H}_3CH(OTHP)$), 1.18 (1.5H, d, *J* 6.4, $C\underline{H}_3CHCO$), 1.26 (1.5H, d, *J* 7.2, $C\underline{H}_3CH(OTHP)$), 1.41-1.83 (6H, m, 3 x $C\underline{H}_2$), 2.52 (0.5H, dq, *J* 6.4, 6.4, $CH_3C\underline{H}CO$), 2.56 (0.5H, dq, *J* 6.4, 6.4, $CH_3C\underline{H}CO$), 3.45 (1H, m, $CH_3C\underline{H}(OTHP)$), 3.72-3.96 (2H, m, $C\underline{H}_2O$), 4.63 (O.5H, dd, *J* 3.5, $OC\underline{H}O$), 4.74 (O.5H, dd, *J* 3.5, $OC\underline{H}O$), 9.74 (0.5H, d, *J* 2.4, $C\underline{H}O$), 9.77 (0.5H, d, *J* 2.4, $C\underline{H}O$); δ_C 16.58, 19.30, 19.78, 21.47, 25.38, 30.70, 62.86 ($CH_3\underline{C}H(OTHP)$), 62.97 ($\underline{C}H_2O$), 99.79 ($O\underline{C}HO$), 203.41 ($\underline{C}HO$) *m/z* (CI) 101 ((M-84)+, 6%), 93 (4), 86 (32), 85 (100), 84 (53), 73 (13), 69 (11), and 57 (53).

N.B. The Baker's yeast reduction gave ethyl (3*S*)-3-hydroxybutanoate in 70% e.e. Therefore, this compound is only, at best, synthesised in 70% e.e.

S-2-(Acetylamino)ethyl (2*E*,4*S*,5*S*)-5-(1'-tetrahydropyran-2-yl)oxy-4-methylhex-2-enethioate (189) (N-acetylcysteamine thioester of (2*E*,4*S*,5*S*)-5-(1'-tetrahydropyran-2-yl)oxy-4-methylhex-2-enoic acid)

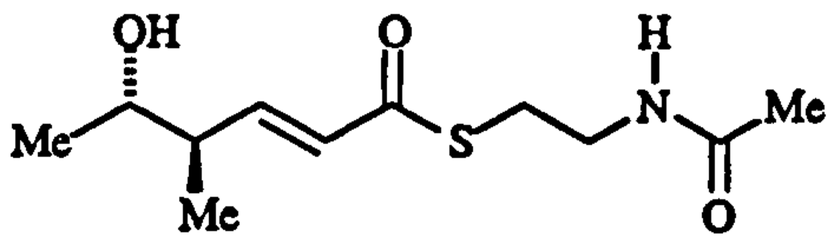


(189)

To (2*S*,3*S*)-3-(1'-tetrahydropyran-2-yl)oxy-2-methylbutanal (188) (0.16 g, 0.86 mmol) was added THF (20 cm^3). The N-acetylcysteamine thioester (151) of triphenylphosphorane acetate (0.4 g, 0.95 mmol) was added, and the reaction mixture was heated to reflux, under a nitrogen atmosphere, for 4 days. The solvent was removed *in vacuo*. Purification by flash column chromatography (SiO_2, 10% ethyl acetate/petroleum ether 40-60 °C) yielded the **S-2-(acetylamino)ethyl (2*E*,4*S*,5*S*)-5-(1'-tetrahydropyran-2-yl)oxy-4-methylhex-2-enethioate (189)** (0.26 g, 92%) as a colourless oil (R_f=0.35). υ_{max} 3269, 1720, 1664 cm^{-1}; δ_H 1.04 (1.5H, d, *J* 5.0, C<u>H</u>$_3$CH), 1.10 (1.5H, d, *J* 6.2, C<u>H</u>$_3$CH(OTHP)), 1.12 (1.5H, d, *J* 5.0, C<u>H</u>$_3$CH), 1.18 (1.5H, d, *J* 6.2, C<u>H</u>$_3$CH(OTHP)), 1.41-1.90 (6H, m, 3 x C<u>H</u>$_2$), 1.97 (3H, s, C<u>H</u>$_3$CON), 2.43 (1H, m, CH$_3$C<u>H</u>CH=CH), 3.09 (2H, t, *J* 6.2, C<u>H</u>$_2$S), 3.46 (2H, t, *J* 6.2, C<u>H</u>$_2$NH), 3.65 (1H, m, CH$_3$C<u>H</u>(OTHP)), 3.75-3.96 (2H, m, C<u>H</u>$_2$O), 4.60 (O.5H, dd, *J* 3.5, OC<u>H</u>O), 4.73 (O.5H, dd, *J* 3.5, OC<u>H</u>O), 6.05 (1H, br s, N<u>H</u>), 6.13 (0.5H, dd, *J* 15.4, 0.5, CH=C<u>H</u>COS), 6.15 (0.5H, dd, *J* 15.4, 0.5, CH=C<u>H</u>COS), 6.92 (0.5H, dd, *J* 15.4, 5.5, <u>C</u>H=CHCOS), 6.97 (0.5H, dd, *J* 15.4, 5.5, C<u>H</u>=CHCOS); *m/z* (CI) 307 (28%), 279 (100), 228 (10), 201 (16), 127 (8), 120 (40), 109 (43), and 60 (14). Found $(M\text{-}OTHP)^+$, 228.1063 $C_{11}H_{18}NO_2S$ requires $(M\text{-}OTHP)^+$, 228.1058.

N.B. The Baker's yeast reduction gave ethyl (3*S*)-3-hydroxybutanoate in 70% e.e. Therefore, this compound is only, at best, synthesised in 70% e.e.

S-2-(acetylamino)ethyl (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enethioate (170) (N-acetylcysteamine thioester of (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enoic acid)



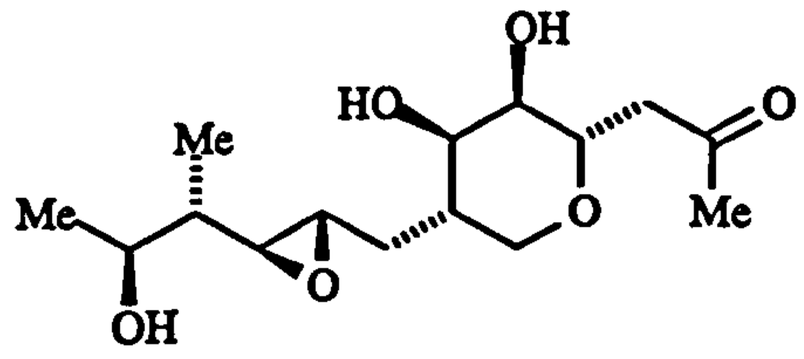
(170)

To a solution of the N-acetylcysteamine thioester (189) of (2*E*,4*S*,5*S*)-5-(1'-tetrahydropyran-2-yl)oxy-4-methylhex-2-enoic acid (0.01 g, 0.03 mmol) in freshly distilled methanol (10 cm^3) was added a catalytic amount of amberlyst. The resulting solution was then stirred at room temperature for 18 hours. The mixture was filtered, and then the solvent was concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 25% ethyl acetate, petroleum ether 40-60 °C) yielded the **S-2-(acetylamino)ethyl (2*E*,4*S*,5*S*)-5-hydroxy-4-methylhex-2-enethioate (170)** (0.002 g, 27%) as a colourless oil (R_f=0.3). υ_{max} 3346, 1720, 1681, 1662, 1567cm^{-1}; δ_H 1.10 (3H, d, *J* 6.8, C<u>H</u>$_3$CH(OH)), 1.21 (3H, d, *J* 6.8, C<u>H</u>$_3$CH), 1.97 (3H, s, C<u>H</u>$_3$CON), 2.36 (1H, m, CH$_3$C<u>H</u>CH=CH), 3.10 (2H, t, *J* 6.4, C<u>H</u>$_2$S), 3.47 (2H, t, *J*

6.4, C<u>H</u>$_2$NH), 3.75 (1H, m, CH$_3$C<u>H</u>(OH)), 5.95 (1H, br s, N<u>H</u>), 6.15 (1H, dd, *J* 15.4, 1, CH=C<u>H</u>COS), 6.92 (1H, dd, *J* 15.4, 7.8, <u>C</u>H=CHCOS); δ_C 15.51 (<u>C</u>H$_3$CH(OH)), 20.92 (<u>C</u>H$_3$CHCH=CH), 23.27 (CH$_2$S), 28.41 (CH$_2$NH), 39.77 (<u>C</u>H$_3$CON), 44.15 (CH$_3$<u>C</u>HCH=CH), 70.81 (CH$_3$<u>C</u>H(OH)), 128.99 (CH=<u>C</u>HCOS), 151.06 (<u>C</u>H=CHCOS), 170.64 (<u>C</u>ON), 190.46 (<u>C</u>OS); *m/z* (CI) 246 (MH$^+$, 71%), 228 (10), 127 (100), 120 (79), 109 (37), 86 (22), 83 (60), and 55 (30). Found MH$^+$, 246.1166 $C_{11}H_{20}NO_3S$ requires MH$^+$, 246.1164.

N.B. The Baker's yeast reduction gave ethyl (3*S*)-3-hydroxybutanoate in 70% e.e. Therefore, this compound is only, at best, synthesised in 70% e.e.

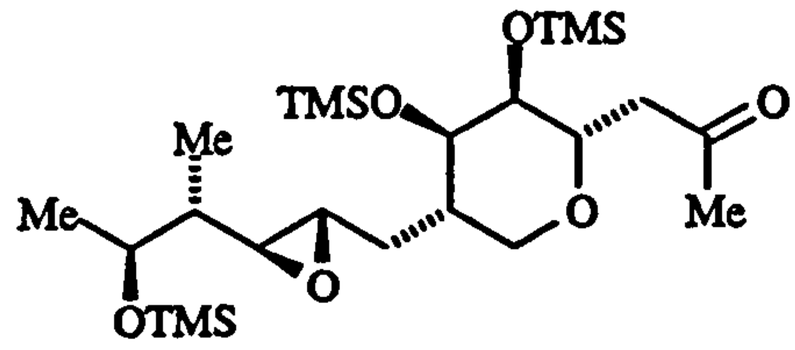
(2*S*,3*R*,4*R*,5*S*)-3,4-hydroxy-5-((2*S*,3*S*,4*S*,5*S*)-2,3-Epoxy-5-hydroxy-4-methylhexyl)tetrahydropyran-2-ylacetone (Methyl Ketone (196))[49]



(196)

Methyl pseudomonate (197) (0.5 g, 0.95 mmol) was dissolved in methanol (8 cm^3), pyridine (2 drops) was added, and ozonised oxygen was bubbled into the solution at -78 °C until it remained persistently blue. Any excess ozone was then blown off by a flow of nitrogen at -78 °C, until colourless. Triethyl phosphite (0.3 cm^3) was then added, and the reaction mixture was warmed slowly to room temperature. The solvent was removed *in vacuo*. Purification by flash column chromatography (SiO_2, 20% ethyl acetate, petroleum ether 40-60 °C) yielded the methyl ketone (196) (0.18 g, 63%) as a colourless oil (R_f=0.3). $[\alpha]_D^{20}$ +10.2 (*c* 2.0, $CHCl_3$), [lit.,[49] +11.9 (*c* 1.0, $CHCl_3$)]; υ_{max} 1708 cm^{-1}; δ_H 0.90 (3H, d, *J* 7.2, 17-<u>H</u>), 1.20 (3H, d, *J* 6.4, 14-<u>H</u>), 1.30 (1H, m, 12-<u>H</u>), 1.65 (2H, m, 9-<u>H</u>), 2.00 (1H, m, 8-<u>H</u>), 2.19 (3H, s, 15-<u>H</u>), 2.57-2.83 (4H, m, 4-<u>H</u>, 11-<u>H</u>, 10-<u>H</u>), 3.07 (3H, br s, O<u>H</u>), 3.50-3.60 (2H, m, 6-<u>H</u>, 16eq-<u>H</u>), 3.64-3.97 (3H, m, 16ax-<u>H</u>, 7-<u>H</u>, 13-<u>H</u>, 5-<u>H</u>); δ_C 12.58 (<u>C</u>-17), 20.65 (<u>C</u>-14), 30.84 (<u>C</u>-15), 31.44 (<u>C</u>-9), 39.57 (<u>C</u>-8), 42.62 (<u>C</u>-12), 46.59 (<u>C</u>-4), 55.54 (<u>C</u>-10), 61.06 (<u>C</u>-11), 65.41 (<u>C</u>-16), 68.77 (<u>C</u>-6), 69.98 (<u>C</u>-7), 71.10 (<u>C</u>-13), 72.75 (<u>C</u>-5), 208.91 (<u>C</u>-3); *m/z* 302 (M$^+$, 1%), 284 (1), 269 (1), 241 (4), 227 (11), 154 (60), 112 (37), and 83 (100).

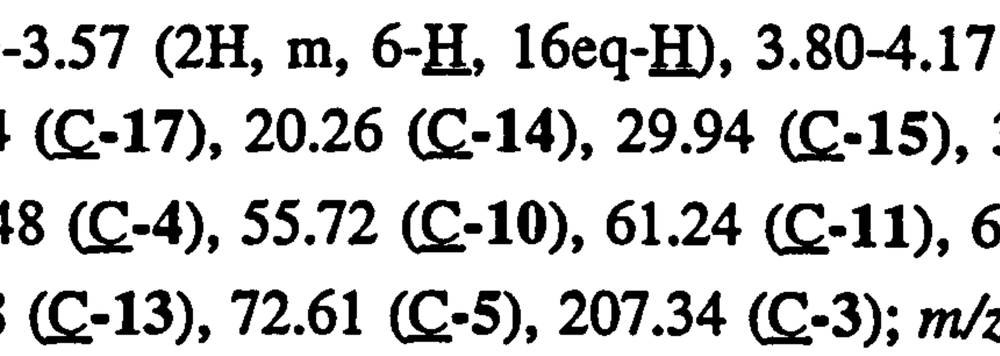
(2*S*,3*R*,4*R*,5*S*)-3,4-trimethylsilyloxy-5-((2*S*,3*S*,4*S*,5*S*)-2,3-Epoxy-5-trimethylsilyloxy-4-methylhexyl)tetrahydropyran-2-ylacetone (201) (Protected ketone (201))[119]



(201)

Trimethylsilyl chloride (0.14 cm^3, 1.06 mmol) was added to the methyl ketone () (0.1 g, 0.33 mmol) in THF (30 cm^3) and triethylamine (0.15 cm^3, 1.07 mmol), and a catalytic amount of 4-dimethylaminopyridine. The reaction mixture was stirred at room temperature for 4 hours. The solution was then filtered, in order to remove triethylamine hydrochloride, and was then concentrated *in vacuo*. The residue was then taken up in diethyl ether (30 cm^3), the solution was filtered, and the solvent removed *in vacuo* to yield the protected ketone (201) (0.12 g, 70%) as a colourless, sticky gum. This was used without further purification. $[\alpha]_D^{20}$ -11.1 (*c* 7.9, $CHCl_3$); δ_H 0.0 (9H, s, $OSi(C\underline{H}_3)_3$), 0.90 (3H, d, *J* 7.0, 17-<u>H</u>), 1.20 (3H, d, *J* 6.24, 14-<u>H</u>), 1.38 (1H, m, 12-<u>H</u>), 1.57 (2H, m, 9-<u>H</u>), 1.79 (1H, m, 8-<u>H</u>), 2.20 (3H, s, 15-<u>H</u>), 2.35-2.75 (4H, m, 4-<u>H</u>, 11-<u>H</u>, 10-<u>H</u>), 3.39-3.57 (2H, m, 6-<u>H</u>, 16eq-<u>H</u>), 3.80-4.17 (4H, m, 16ax-<u>H</u>, 7-<u>H</u>, 13-<u>H</u>, 5-<u>H</u>); δ_C 12.54 (<u>C</u>-17), 20.26 (<u>C</u>-14), 29.94 (<u>C</u>-15), 31.43 (<u>C</u>-9), 41.72 (<u>C</u>-8), 42.64 (<u>C</u>-12), 46.48 (<u>C</u>-4), 55.72 (<u>C</u>-10), 61.24 (<u>C</u>-11), 65.15 (<u>C</u>-16), 68.00 (<u>C</u>-6), 69.88 (<u>C</u>-7), 71.18 (<u>C</u>-13), 72.61 (<u>C</u>-5), 207.34 (<u>C</u>-3); *m/z* (CI) 519 (MH^+, 8%), 518 (M^+, 1), 295 (30), 117 (100), 91 (50), 75 (79), 73 (93), and 59 (43).

Ethyl 4-[(2*S*,3*R*,4*R*,5*S*)-3,4-trimethylsilyloxy-5-((2*S*,3*S*,4*S*,5*S*)-2,3-Epoxy-5-trimethylsilyloxy-4-methylhexyl)tetrahydropyran-2-yl]-3-methylbut-(2*E*)-enoate (202)

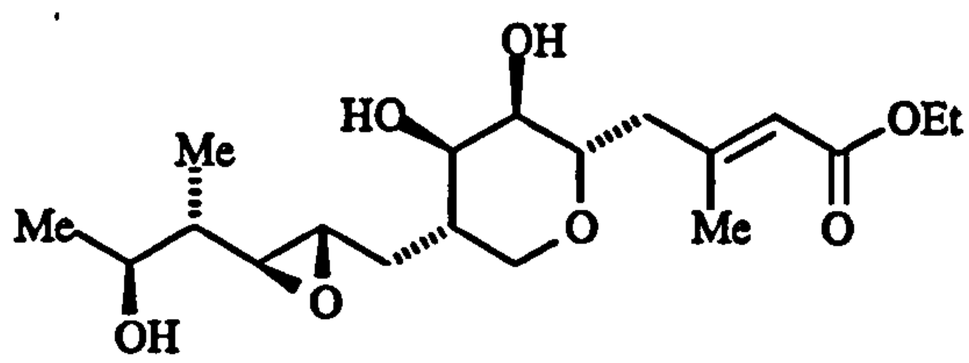


(202)

Sodium hydride (60% dispersion in oil, 1.28 g, 32.0 mmol) was stirred in freshly distilled THF (15 cm^3) at 0 °C under a nitrogen atmosphere. Ethyl diethylphosphonoacetate (162) (9.15 g, 40.8 mmol) in THF (15 cm^3) was added

dropwise, at 0^0C, over 15 minutes, after which time a pale yellow solution was present. The mixture was stirred for a further 15 minutes, after which time the protected ketone (201) (2.14 g, 4.13 mmol) in distilled THF (15 cm^3) was added dropwise over 10 minutes. The reaction mixture was allowed to warm to room temperature overnight, and then poured into an ice-cold phosphate buffer (K_2HPO_4) (0.1M, 100 cm^3), and stirred at room temperature. The mixture was then extracted with ethyl acetate (4 x 50 cm^3), and the combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 30% ethyl acetate, petroleum ether 40-60 °C) yielded **ethyl 4-[(2*S*,3*R*,4*R*,5*S*)-3,4-trimethylsilyloxy-5-((2*S*,3*S*,4*S*,5*S*)-2,3-Epoxy-5-trimethylsilyloxy-4-methylhexyl)tetrahydropyran-2-yl]-3-methylbut-(2*E*)-enoate (202)** (0.96 g, 40%) as a colourless oil (R_f=0.6). $[\alpha]_D^{20}$ -11.7 (*c* 1.68, $CHCl_3$); υ_{max} 1722, 1649 cm^{-1}; δ_H 0.0 (9H, s, OSi($C\underline{H}_3$)$_3$), 0.86 (3H, d, *J* 7.0, 17-$\underline{H}$), 1.16 (3H, d, *J* 6.2, 14-$\underline{H}$), 1.24 (3H, t, *J* 7.0, $C\underline{H}_3CH_2O$), 1.35 (1H, m, 12-$\underline{H}$), 1.56 (2H, m, 9-$\underline{H}$), 1.62-1.92 (1H, m, 8-$\underline{H}$), 2.16 (3H, s, 15-$\underline{H}$), 2.48-2.68 (4H, m, 4-$\underline{H}$, 11-$\underline{H}$, 10-$\underline{H}$), 3.31-3.49 (2H, m, 6-$\underline{H}$, 16eq-$\underline{H}$), 3.75-3.89 (4H, m, 16ax-$\underline{H}$, 7-$\underline{H}$, 13-$\underline{H}$, 5-$\underline{H}$), 4.12 (2H, t, *J* 7.0, $CH_3C\underline{H}_2O$), 5.71 (1H, s, 2-$\underline{H}$); δ_C 0 (OSi($\underline{C}H_3$)$_3$), 12.55 ($\underline{C}$-17), 14.36 ($\underline{C}H_3CH_2O$), 18.80 ($\underline{C}$-15), 20.90 ($\underline{C}$-14), 32.14 ($\underline{C}$-9), 41.93 ($\underline{C}$-8), 42.75 ($\underline{C}$-12), 43.19 ($\underline{C}$-4), 55.35 ($\underline{C}$-10), 59.38 ($CH_3\underline{C}H_2O$), 61.40 ($\underline{C}$-11), 65.50 ($\underline{C}$-16), 70.40 ($\underline{C}$-6), 70.78 ($\underline{C}$-7), 73.10 ($\underline{C}$-13), 73.91 ($\underline{C}$-5), 117.14 ($\underline{C}$-2), 157.15 ($\underline{C}$-3), 166.45 ($\underline{C}$-1); *m/z* (CI) 589 (MH^+, 100%), 588 (M^+, 4), 542 (16), 518 (2), 371 (18), 117 (76.2), and 73 (27.3). Found MH^+, 589.3411 $C_{28}H_{57}Si_3O_7$ requires MH^+, 589.3412.

Ethyl 4-[(2*S*,3*R*,4*R*,5*S*)-3,4-hydroxy-5-((2*S*,3*S*,4*S*,5*S*)-2,3-Epoxy-5-hydroxy-4-methylhexyl)tetrahydropyran-2-yl]-3-methylbut-2(*E*)-enoate (204) (Ethyl monate A (204))[117]

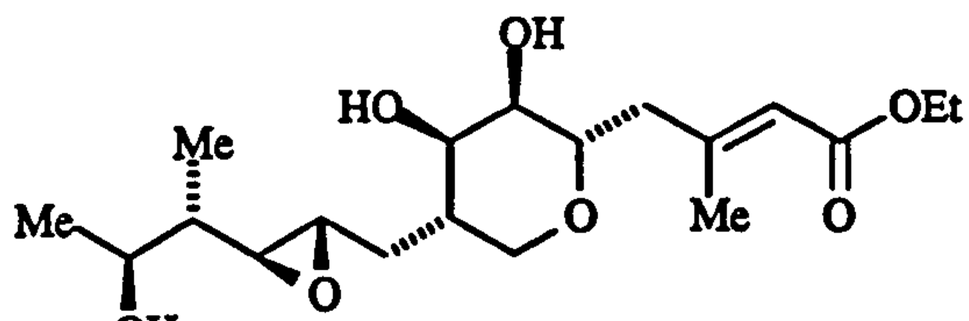


(204)

To a stirred solution of ethyl 6,7,13-trimethylsilyloxymonate A (202) (0.60 g, 1.02 mmol) in freshly distilled THF (30 cm^3) was added HCl (0.4M, 6 cm^3) in one portion, and stirred for two minutes exactly from the time of addition. This solution was then immediately quenched with saturated sodium hydrogen carbonate (6 cm^3), and the mixture was stirred for 5 minutes, and then extracted with ethyl acetate (3 x 25 cm^3). The combined organic extracts were dried with sodium sulphate, filtered and concentrated *in*

vacuo to yield ethyl monate A (204) (0.32 g, 84%) as an opaque, sticky gum. $[\alpha]_D^{20}$ -2.3 (*c* 2.5, $CHCl_3$), [lit.,[117] -1.4 (*c* 1.8, $CHCl_3$)]; υ_{max} 3406, 1708, 1656 cm^{-1}; δ_H 0.94 (3H, d, *J* 7.0, 17-H), 1.22 (3H, d, *J* 6.4, 14-H=), 1.27 (3H, t, *J* 7.0, CH_3CH_2O), 1.74 (3H, m, 12-H, 9-H), 2.02 (1H, m, 8-H), 2.21 (3H, s, 15-H), 2.50-2.84 (4H, m, 4-H, 11-H, 10-H), 3.52-3.58 (2H, m, 6-H, 16eq-H), 3.71-3.93 (4H, m, 16ax-H, 7-H, 13-H, 5-H), 4.14 (2H, q, *J* 7.0, CH_3CH_2O) 6.10 (1H, s, 2-H); δ_C 12.76 (C-17), 14.32 (CH_3CH_2O), 19.05 (C-15), 20.83 (C-14), 31.68 (C-9), 39.53 (C-8), 42.83 (C-12), 42.95 (C-4), 55.56 (C-10), 59.62 (CH_3CH_2O), 61.37 (C-11), 65.34 (C-16), 69.02 (C-6), 70.38 (C-7), 71.46 (C-13), 74.77 (C-5), 117.66 (C-2), 156.64 (C-3), 166.68 (C-1); *m/z* (CI) 373 (MH^+, 74%), 372 (M^+, 2), 327 (84), 309 (100), 291 (58), 279 (43), 227 (72), and 209 (38).

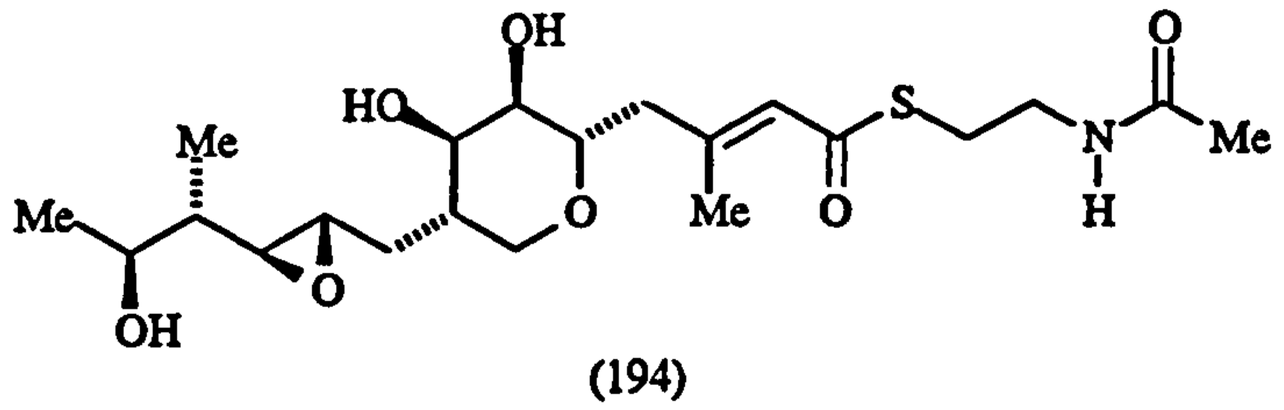
Treatment of ethyl 6,7,13-trimethylsilyloxymonate A (202) with sodium hydroxide



(204)

To a stirred solution of ethyl 6,7,13-trimethylsilyloxymonate A (202) (0.52 g, 0.88 mmol) was added sodium hydroxide solution (2M, 0.49 cm^3, 0.98 mmol),. The reaction mixture was stirred at room temperature overnight, and was quenched with hydrochloric acid (1M, 5 cm^3). This was then extracted with ethyl acetate (5 x 25 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield ethyl monate A (204) (0.28 g, 85%) as an opaque, sticky gum. Spectral data as before.

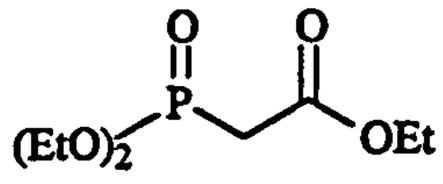
S-2-(Acetylamino)ethyl 4-[(2*S*,3*R*,4*R*,5*S*)-3,4-hydroxy-5-((2*S*,3*S*,4*S*,5*S*)-2,3-Epoxy-5-hydroxy-4-methylhexyl)tetrahydropyran-2-yl]-3-methylbut-(2*E*)-enethioate (194) (N-acetylcysteamine thioester of 4-[(2*S*,3*R*,4*R*,5*S*)-3,4-hydroxy-5-((2*S*,3*S*,4*S*,5*S*)-2,3-Epoxy-5-hydroxy-4-methylhexyl)tetrahydropyran-2-yl]-3-methylbut-(2*E*)-enoic acid (N-acetylcysteamine thioester of monic acid A))



(194)

Monic acid A (50) (1.81 g, 5.26 mmol) was added to dichloromethane (50 cm^3), under a nitrogen atmosphere. Freshly prepared N-acetylcysteamine (73) (1.01 g, 8.49 mmol) in dichloromethane (4 cm^3), followed by dicyclohexylcarbodiimide (83) (1.16 g, 5.62 mmol) in dichloromethane (4 cm^3), and 4-dimethylaminopyridine (84) (0.06 g, 0.49 mmol) in dichloromethane (2 cm^3) was added. The reaction was left to warm to room temperature overnight. After 24 h., the solution was washed with saturated ammonium chloride (40 cm^3), and was extracted with dichloromethane (2 x 25 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, 25% ethyl acetate/ petroleum ether 40-60 °C) yielded the **S-2-(acetylamino)ethyl 4-[(2*S*,3*R*,4*R*,5*S*)-3,4-hydroxy-5-((2*S*,3*S*,4*S*,5*S*)-2,3-Epoxy-5-hydroxy-4-methylhexyl)tetrahydropyran-2-yl]-3-methylbut-(2*E*)-enethioate (194)** (1.44 g, 62%) as an opaque, sticky gum. $[\alpha]_D^{20}$ -2.1 (*c* 3.3, $CHCl_3$); υ_{max} (*nujol*) *3400, 1687, 1653* cm^{-1}; δ_H 0.94 (3H, d, *J* 7.0, 17-H), 1.22 (3H, d, *J* 6.2, 14-H), 1.30 (1H, m, 12-H), 1.72 (2H, m, 9-H), 1.98 (3H, s, CH_3CON), 2.07 (1H, m, 8-H), 2.22 (4H, m, 4ax-H, 15-H), 2.61 (1H, m, 4eq-H), 2.71 (1H, m, 11-H), 2.81 (1H, m, 10-H), 3.07 (2H, t, *J* 6.5, CH_2S), 3.45 (2H, q, *J* 6.5, CH_2NH), 3.54-3.64 (2H, m, 6-H, 16eq-H), 3.70-3.96 (4H, m, 16ax-H, 7-H, 13-H, 5-H), 6.10 (1H, s, 2-H), 6.17 (1H, br s, NH); δ_C 12.67 (C-17), 20.32 (C-15), 20.86 (C-14), 23.18 (CH_2NH), 28.48 (CH_2S), 31.65 (C-9), 39.72 (CH_3CON), 39.91 (C-8), 42.76 (C-12), 42.83 (C-4), 55.56 (C-10), 61.21 (C-11), 65.40 (C-16), 68.83 (C-6), 70.39 (C-7), 71.31 (C-13), 74.89 (C-5), 124.17 (C-2), 155.50 (C-3), 170.64 (CON), 189.41 (COS); *m/z* (CI) 446 (MH^+, 33%), 444 (1), 426 (72), 327 (73), 309 (78), 291 (56), 120 (100), and 60 (48). Found MH^+, 446.2204 $C_{21}H_{36}NO_7S$ requires MH^+, 446.2213.

Ethyl diethylphosphonoacetate (162)[141]

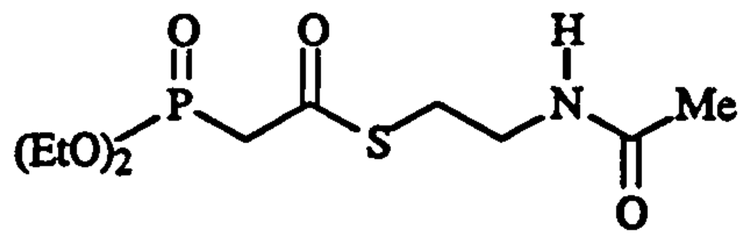


(162)

Triethylphosphite (0.86 cm^3, 5.00 mmol) was added to a solution of ethyl bromoacetate (0.70 g, 4.19 mmol) in dry toluene (10 cm^3), and the reaction mixture was heated to reflux for 24 h. The solvent was then removed *in vacuo* to yield ethyl diethylphosphonoacetate (162) (0.74 g, 79%) as a colourless oil. This was used without further purification. δ_H 1.29 (3H, t, *J* 7.2, $C\underline{H}_3CH_2O$), 1.35 (6H, t, *J* 7.2, 2 x $(C\underline{H}_3CH_2O)_2P$), 2.96 (2H, d, *J* 21.4, $C\underline{H}_2P$), 4.16 (4H, q, *J* 7.2, 2 x $(CH_3C\underline{H}_2O)_2P$), 4.20 (2H, q, *J* 7.2, $CH_3C\underline{H}_2O$).

S-2-(Acetylamino)ethyl 2-diethylphosphonoethanethioate (190)

(N-acetylcysteamine thioester of diethylphosphonoacetic acid)



(190)

To a solution of the N-acetylcysteamine thioester (152) of bromoacetic acid (1.12 g, 4.70 mmol) in dry toluene (10 cm^3) was added triethylphosphite (1.00 cm^3, 5.83 mmol). The reaction mixture was heated to reflux for 24 h. The solvent was then removed *in vacuo* to yield **S-2-(acetylamino)ethyl 2-diethylphosphonoethanethioate (190)** (1.10 g, 79%) as a pale yellow oil. This was used without further purification. υ_{max} 3293, 1655, 1249 cm^{-1}; δ_H 1.36 (6H, t, *J* 7.2, 2 x $(C\underline{H}_3CH_2O)_2P$), 1.98 (3H, s, $C\underline{H}_3CON$), 3.03 (2H, t, *J* 6.2, $C\underline{H}_2S$), 3.25 (2H, d, *J* 21.4, $C\underline{H}_2P$), 3.41 (2H, q, *J* 6.2, $C\underline{H}_2NH$), 4.16 (4H, q, *J* 7.2, 2 x $(CH_3C\underline{H}_2O)_2P$), 6.51 (1H, br s, $N\underline{H}$); δ_C 16.23 ($\underline{C}H_3CH_2O$), 23.04 ($\underline{C}H_2NH$), 29.47 ($\underline{C}H_2S$), 39.01 ($\underline{C}H_3CON$), 42.07 ($\underline{C}H_2P$), 62.96 ($CH_3\underline{C}H_2O$), 170.70 ($\underline{C}ON$), 196.90 ($\underline{C}OS$); *m/z* (CI) 298 (MH^+, 100%), 279 (30), 179 (10), 120 (10), and 118 (5). Found MH^+, 298.0892 $C_{10}H_{21}NO_5PS$ requires MH^+, 298.0880.

4.3 Experimental for the culture work discussed in Chapter 3

Agar Slant Preparation

The following ingredients were added in order, as follows:

Constituents	Weight (g/litre)
Spray dried yeatex	2.28
D-glucose	1.10
Na_2HPO_4	5.00
$(NH_4)_2SO_4$	5.00
Agar No.3	30.0

Table 4.1: Constituents of the 1/10 6sp agar slant medium.

The required volume of water was added, and the pH was then adjusted to pH 7.0. 7 cm^3 was dispensed into each of ten test tubes. They were then autoclaved for 15 minutes at 121 °C, after which time the test tubes were laid on their sides and left to set for 24 hours. The slopes were then inoculated with a 10µl loopful of *Pseudomonas fluorescens* NCIB 10586, and left to grow at 25 °C for 5 days, after which time they were stored at 4 °C, and were viable for three months.

Culturing of *Pseudomonas fluorescens*

(a) Preparation of primary stage medium

The following ingredients were added in order, as follows:

Constituents	Weight (g/litre)
Spray dried yeatex	22.8
D-glucose	1.10
NaH_2PO_4	5.00
$(NH_4)_2SO_4$	5.00
Anti-foam	0.5 cm^3/litre

Table 4.2: Constituents of the primary stage medium.

The required volume of water was added, and the pH was then adjusted to pH 7.0. 50

cm^3 was then dispensed into one 250 cm^3 conical flask, and this flask was autoclaved at 121 °C for 15 minutes. The flask was then inoculated with 10 µl loopful of *Pseudomonas fluorescens* NCIB 10586, and was shaken at 25 °C for 24 hours at 240rpm.

(b) Preparation of final stage medium

The following ingredients were added in order, as follows:

Constituents	Weight (g/litre)
D-glucose	100
Arkasoy 50	20
Spray dried corn steep liquor	2.5
$(NH_4)_2SO_4$	9
$CaCO_3$	20
$MgSO_4$	0.5
NaH_2PO_4	2.5
Anti-foam	0.5 cm^3/litre

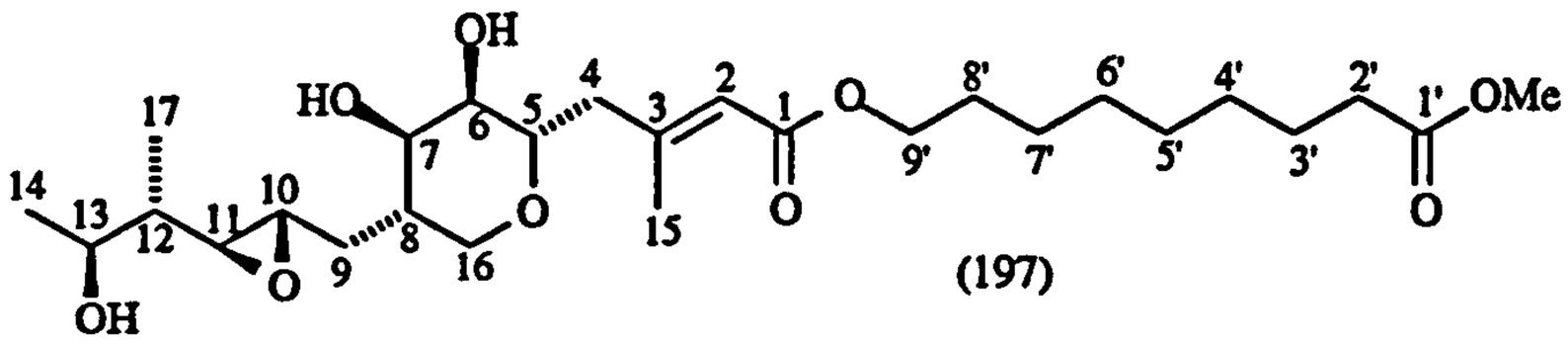
Table 4.3: Constituents of the final stage medium.

The required volume of water was added, and the pH was then adjusted to pH 7.0. 25 cm^3 was then dispensed into each of ten baffled 250 cm^3 conical flasks, and these flasks were autoclaved at 121 °C for 15 minutes. The ten flasks were then inoculated by transferring 1 cm^3 of the primary medium to each of the ten flasks, and these flasks were shaken at 22 °C for 5 days at 240rpm.

HPLC Assay

An aliquot (1 cm^3) was taken from a flask, so as to determine the titre of pseudomonic acid present in the medium at that time. The samples were micro-centrifuged before analysis by HPLC. The supernatant obtained from micro-centrifugation was then diluted ten-fold (*i.e.* 0.05 cm^3 of supernatant, and 0.45 cm^3 of distilled water). The pseudomonic acid content was determined by using reverse phase HPLC, using a C-18 Whatman perisphere column, injecting 25 µl, and using a solvent system of ammonium acetate (0.58 g/litre), 'HPLC grade' methanol (200 cm^3), and distilled water (800 cm^3). The flow rate was 1.5 cm^3 min^{-1}, with the peaks being monitored at 233nm, the wavelength of the chromaphore of pseudomonic acid. Pseudomonic acid A was observed to have a retention time of 6.5 minutes with this solvent system.

Isolation of methyl pseudomonate (197)[48]



(197)

The combined secondary media were centrifuged (10000 g, 2 °C, 15 minutes) and the supernatant was acidified to pH 4.5, saturated with sodium chloride and extracted with ethyl acetate (5 x 150 cm^3). The combined organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. The orange oil was taken up in 5 cm^3 of methanol, and treated with an excess of ethereal diazomethane. After one hour the excess diazomethane was blown off with nitrogen, and the mixture was concentrated *in vacuo*. Purification by preparative TLC (2.5% methanol in ethyl acetate) yielded methyl pseudomonate (197) as a colourless oil (R_f=0.3). $[\alpha]_D^{20}$ -8.2 (*c* 2.5, $CHCl_3$); [lit.,[48] -9.0 (*c* 1.5, $CHCl_3$)]; υ_{max} (thin film) 3310, 1750, 1720, 1655 cm^{-1}. The ^1H and ^{13}C nmr data can be found in Tables 3.1 and 3.2 respectively

Preparation of ethereal diazomethane[142,143]

Potassium hydroxide (14 g) in ethanol (50 cm^3) and water (15 cm^3) was heated in a water bath at 65 °C. To this solution was added diazald (43 g) in diethyl ether (250 cm^3) from a separating funnel. The mixture was then added at such a rate that a gentle reflux led to slow distillation of the yellow distillate into an ice-cooled conical flask. Once complete, the remaining mixture was quenched with acetic acid (10%) in diethyl ether. Diazomethane was then stored in a freezer until required.

Methylation of pseudomonic acid

The combined secondary media were centrifuged (10000 g, 2 °C, 15 minutes) and the supernatant was acidified to pH 4.5, saturated with sodium chloride and extracted with ethyl acetate (5 x 150 cm^3). The organic extracts were dried over magnesium sulphate, filtered and concentrated *in vacuo*. The resulting orange oil was taken up in methanol (5 cm^3), and treated with an excess of ethereal diazomethane. To test there was an excess of diazomethane, a few drops of acetic acid was added. Evolution of nitrogen indicated there was an excess. To remove the excess, the solution was quenched with acetic acid, until no more nitrogen evolved. Nitrogen was bubbled through the solution, and the resulting mixture was then concentrated *in vacuo* to yield crude methyl pseudomonate (197).

Growth production study

An aliquot (1 cm^3) was taken from at least two flasks from the secondary media every two hours, commencing 10 hours after inoculation. The pH of the combined media was taken for each sample. Analysis by HPLC then followed, using the same assay, as previously described.

Time(hours)	Titre(mg/ cm^3)	pH
0	0.000	7.00
12	0.024	6.80
15	0.094	5.90
18	0.162	5.82
21	0.233	5.73
24	0.274	5.66
27	0.355	5.61
30	0.316	5.55
33	0.380	5.50
36	0.324	5.57
39	0.446	5.53
42	0.335	5.45
45	0.364	5.29
48	0.329	5.19
50	0.300	5.12
63	0.383	7.82
66	0.363	7.75
72	0.381	7.67
120	0.388	8.40

Table 4.4: Growth production results with NCIB 10586.

Incorporation studies with *Pseudomonas fluorescens* PF3/R

(a) Sodium [1-^{13}C]acetate

Primary stage and secondary stage media were inoculated as described earlier for NCIB 10586. A 5 cm^3 solution (sterile water), containing sodium [1-^{13}C]acetate (250mg) was prepared. 0.5 cm^3 was added to each of 10 flasks, 43 hours after inoculation. The cells were harvested as usual. HPLC analysis gave a value of 11.81%, of the value of the control flask. Methyl pseudomonate (31mg) was isolated. The ^{1}H & ^{13}C nmr spectrum was identical to that of authentic methyl pseudomonate, showing that there was no significant incorporation.

(b) Sodium [2-^{13}C]acetate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [2-^{13}C] acetate (250mg) was added. The cells were harvested as usual. HPLC analysis gave a value of 11.64% of the value of the control flask. Methyl pseudomonate (14mg) was isolated. The ^{1}H & ^{13}C nmr spectrum was identical to that of authentic methyl pseudomonate, showing that there was no significant incorporation.

(c) Sodium [1,2-$^{13}C_2$]acetate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [1,2-$^{13}C_2$]acetate (250mg) was added. The cells were harvested as usual. HPLC analysis gave a value of 12.00%, of the value of the control flask. Methyl pseudomonate (25mg) was isolated. The ^{1}H & ^{13}C nmr spectrum was identical to that of authentic methyl pseudomonate, showing that there was no significant incorporation.

(d) Sodium [1-^{13}C]propionate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [1-^{13}C] propionate (250mg) was added. The cells were harvested as usual. HPLC analysis gave a value of 13.56%, of the value of the control flask. Methyl pseudomonate (30mg) was isolated. The ^{1}H & ^{13}C nmr spectrum was identical to that of authentic methyl pseudomonate, showing that there was no significant incorporation.

(e) Sodium [2-^{13}C]propionate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [2-^{13}C] propionate (250mg) was added. The cells were harvested as usual. HPLC analysis gave a

value of 13.69%, of the value of the control flask. Methyl pseudomonate (15mg) was isolated. The ^{1}H & ^{13}C nmr spectrum was identical to that of authentic methyl pseudomonate, showing that there was no significant incorporation.

(f) Sodium [3-^{13}C]propionate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [3-^{13}C] propionate (250mg) was added. The cells were harvested as usual. HPLC analysis gave a value of 15.09%, of the value of the control flask. Methyl pseudomonate (21mg) was isolated. The ^{1}H & ^{13}C nmr spectrum was identical to that of authentic methyl pseudomonate, showing that there was no significant incorporation.

(g) Sodium [1-^{13}C]butyrate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [1-^{13}C] butyrate (250mg) was added. The cells were harvested as usual. HPLC analysis gave a value of 15.12%, of the value of the control flask. Methyl pseudomonate (27mg) was isolated. The ^{1}H & ^{13}C nmr spectrum was identical to that of authentic methyl pseudomonate, showing that there was no significant incorporation.

(h) Disodium [1,2-$^{13}C_2$]malonate

Seed stage and final stage media were inoculated as described earlier. A 5 cm^3 solution (sterile water), containing disodium [1,2-$^{13}C_2$]malonate (250mg) was prepared. 0.5 cm^3 was added to each of 10 flasks, 43 hours after inoculation. However, after shaking the flasks at 250rpm for 6 days, the medium had coagulated. The contents of the ten flasks were then centrifuged. However, it was impossible to spin out the cells, so no methylation of the pseudomonic acid isolated from this feed was carried out.

Incorporation studies with *Pseudomnas fluorescens* NCIB 10586

(a) Sodium [1-^{13}C]acetate

Primary stage and secondary stage media were inoculated as described earlier. A 5 cm^3 solution (sterile water), containing sodium [1-^{13}C]acetate (250mg) was prepared. 0.5 cm^3 was added to each of 10 flasks, 15 hours after inoculation, as a single feed. The cells were harvested as usual. HPLC analysis showed very little pseudomonic acid production, compared with the control flask. Methyl pseudomonate (3mg) was isolated. There was not enough methyl pseudomonate to obtain a meaningful ^{13}C nmr spectrum.

(b) Sodium [2-^{13}C]acetate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [2-^{13}C] acetate (250mg) was added. The cells were harvested as usual. HPLC analysis showed very little pseudomonic acid production, compared with the control flask. Methyl pseudomonate (5mg) was isolated. There was not enough methyl pseudomonate to obtain a meaningful ^{13}C nmr spectrum.

(c) Sodium [1,2-$^{13}C_2$]acetate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [1,2-$^{13}C_2$]acetate (250mg) was added. The cells were harvested as usual. HPLC analysis showed very little pseudomonic acid production, compared with the control flask. Methyl pseudomonate (5mg) was isolated. There was not enough methyl pseudomonate to obtain a meaningful ^{13}C nmr spectrum.

(d) Sodium [1-^{13}C]propionate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [1-^{13}C] propionate (250mg) was added. The cells were harvested as usual. HPLC analysis showed very little pseudomonic acid production, compared with the control flask. Methyl pseudomonate (4mg) was isolated. There was not enough methyl pseudomonate to obtain a meaningful ^{13}C nmr spectrum.

(e) Sodium [2-^{13}C]propionate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [2-^{13}C] propionate (250mg) was added. The cells were harvested as usual. HPLC analysis showed very little pseudomonic acid production, compared with the control flask. Methyl pseudomonate (2mg) was isolated. There was not enough methyl pseudomonate to obtain a meaningful ^{13}C nmr spectrum.

(f) Sodium [3-^{13}C]propionate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [3-^{13}C] propionate (250mg) was added. The cells were harvested as usual. HPLC analysis showed very little pseudomonic acid production, compared with the control flask. Methyl pseudomonate (3mg) was isolated. There was not enough methyl pseudomonate to obtain a meaningful ^{13}C nmr spectrum.

(g) Sodium [1-^{13}C]butyrate

The same method was employed, as for sodium [1-^{13}C]acetate, except sodium [1-^{13}C] butyrate (250mg) was added. The cells were harvested as usual. HPLC analysis showed very little pseudomonic acid production, compared with the control flask. Methyl pseudomonate (2mg) was isolated. There was not enough methyl pseudomonate to obtain a meaningful ^{13}C nmr spectrum.

(h) Sodium [1-^{13}C]acetate

Primary stage and secondary stage media were inoculated as described earlier. A 5 cm^3 solution (sterile water), containing sodium [1-^{13}C]acetate (250mg) was prepared. 0.5 cm^3 was added to each of 10 flasks, 14, 16, an 18 hours after inoculation, as a pulse feed. The cells were harvested as usual. HPLC analysis showed very little pseudomonic acid production, compared with the control flask. Methyl pseudomonate (2mg) was isolated. There was not enough methyl pseudomonate to obtain a meaningful ^{13}C nmr spectrum.

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4.4 References

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